

Where does Europe go from here?

Last weekend's European summit served chiefly to remind its participants that the European Community is far from being a self-consistent legal entity.

LAST weekend's meeting at Strasbourg of the heads of government of the European Community (EC) seems, against recent precedent, to have been well-mannered and even subdued. This is scarcely surprising. Mrs Margaret Thatcher, recently the chief trouble-maker at these occasions, may have had domestic reasons for showing that chauvinism can have a comely face, but all the participants at Strasbourg had plainly taken to heart Mr Mikhail Gorbachev's warning (to the disgraced leader of East Germany) that "you cannot stand in the doorway of history". They had better get on with making the EC into a common market (which it was always supposed to be) because events in Eastern Europe are treading on their heels.

It is a most curious business. The European Community owes its present form to the post-war division of Europe at Yalta. Although the 1950s founders of the community, the Jean Monnets of the times, nursed some soft thoughts that European federalism would, of its nature, be virtuous, they were mostly concerned with the political and economic benefits of economic union: a common market in Western Europe would be a bulwark against both military threats from the East and economic threats from across the Atlantic. The EC would have been created in just this form if it had seemed, in the 1950s, that the Yalta agreement might be as impermanent as it now appears.

Subdued

That explains why last weekend's meeting was subdued. It is hard to summon up enthusiasm, or even the passion required for radical disagreement, if there is a nagging doubt that the whole basis of the argument may be inappropriate. The internal agenda had been widely advertised — to arrange for a revision of the Treaty of Rome so as to allow for monetary union. Although there will be an intergovernmental conference beginning at the end of next year, the revision process will not be complete until 1993. And it will be contentious. The British, for example, will dig in their heels at many points. So, nearer decision time, will the West Germans, fearful that their own strong currency cannot but be harmed by too close an association with those of the rest of Western Europe. Yet a common market without a common currency (and a central bank to support it) makes no sense — and would give the commercial banking system needless profit from the needless

interconversion of national currencies. On this issue, there is no choice, but why not settle in the meantime, or perhaps indefinitely, for the sensible device of letting national currencies coexist with a European currency (see *Nature* 340, 580; 1989).

Last weekend's meeting draws attention to another weakness of the whole EC enterprise — its constitutional lack of a foreign policy. It is true, of course, that foreign ministers of member states can and, when they agree, do issue joint statements of their opinion on important issues, but that is not the same as being able to decide what should be done about emerging problems. The case of East Germany and the prospect of its reunification with West Germany is the tricky question. Chancellor Helmut Kohl preempted common decision by his speech, at the end of last month, describing his reunification plan. He was less respectful of the common EC interest than he should have been. One crucial consideration should have been the need to reassure the Soviet Union that its security arrangements will not be impaired — without which the liberalization of the rest of Eastern Europe will be put in hazard. Another is that the now open German border raises the prospect that the citizenship of EC may be increased by up to 17 million on the say-so of one government. Last weekend's meeting had to say something. What it said was mostly what Kohl himself had neglected to say, but in language that is thoroughly ambiguous.

How can this state of affairs be remedied? Not easily. If it takes an intergovernmental conference and a treaty revision to settle on a common currency and the ending of national idiosyncrasies (called "sovereignty") which that entails, reaching a common understanding on issues in external relations would be even more difficult. Yet there is an urgent need for a common understanding on issues ranging from military procurement to immigration policy. For lack of an understanding on the second issue, this Friday's meeting in Luxembourg to agree on frontier controls between West Germany, France and the Benelux countries is likely to be abortive. But this is simply another way of saying that even the Treaty of Rome embodies the notion that the EC must become a kind of nation-state. The requirement that people are free to live where they choose implies no less. European governments, always unwilling to acknowledge that, are understandably perplexed by the issue now. □



Broadcast news

Britain is about to destabilize a good broadcasting structure to satisfy Tory ideology and Treasury greed.

THE trouble with the lessons of history is that it is easy to get them wrong. Because the fears aroused in Britain in the early 1950s about the arrival of commercial television turned out to be exaggerated, the Conservative government is now laughing away all the warnings about arguments about the imminent destruction of public-service broadcasting.

Let it be said right away that this is no parochial British matter. The high quality of Britain's four-channelled television service, well subsidised either by the BBC's licence fee or commercial television's near-monopoly on television advertising revenue, for many years brightened and sustained the woefully under-financed American Public Broadcasting Service. More recently, with the advent of satellite distribution and cable television in Europe, the four British channels are widely available in Europe. And with Eastern Europe opening up too, this is a poor time, indeed, for the Thatcher government to undermine the national broadcasting excellence.

The main proposal in the Broadcasting Bill unveiled last week is to award commercial television licences to the highest bidder. During the past year since this proposal was announced, critics from all sides, including Mr Rupert Murdoch of News International and the pillars of the British advertising industry, have been begging the government to think again. Some concessions have been wrung from the free-market diehards, led by the Prime Minister, behind the bill. Before being entitled to offer a cash bid, would-be broadcasters must say what kinds of programmes they would offer in "extraordinary circumstances," moreover, even after this passing this "quality threshold", the highest bidder may be turned down. But that's all. Other than that, the licence to broadcast must go to the highest bidder. The temptation to over-bid is therefore built into the plan. The only way this money can be recovered is by reducing spending on programmes — or by letting the company be taken over. As the new licences, which will take effect in 1993, will be accompanied by a lighter regulatory regime — a new agency, the Independent Television Commission, is to replace the present Independent Broadcasting Authority — there will be no obligation to produce programmes for children, for education, for minorities.

Critics of the bill argue that these changes will not only take away excellence from the commercial system (which, contrary to much opinion abroad, is a source of many fine current affairs programmes documentaries) but also weaken the BBC by lowering the quality of its competition. In response, the government scornfully resurrects the fears of the 1950s then that the coming of what is known as ITV would ruin British cultural life. Lord Reith, founder of the BBC, compared it to the Black Death. Instead, as it turned out, competition was good for the

BBC, forcing it to acquire the popular touch, which led to the satiric and comic renaissance of the 1960s.

The 1950s parallel does not hold, however. ITV started out as a "a licence to print money" because it had a monopoly on advertising revenue and stole two-thirds of the audience by giving it game shows. It only turned from offering the cheap diet to viewers in favour of the more expensive one which included serious current affairs, education, music and community affairs, when it was compelled by parliamentary wrath and tough IBA regulation. However unwillingly, it could obey the regulatory strictures because it had the money to do so. The 1990s are not going to be like that. With the many new channels of satellite and cable competing for advertisements, commercial television is going to be short of money. And it will be more loosely regulated: a recipe for lower standards. The government conveniently chooses to ignore parallels closer in time. In Australia, deregulation of commercial television two years ago led first to sales of licences at vastly inflated prices, followed by takeovers at fractions of their original value. Serious programmes of science, business and arts have disappeared. The Australian Broadcasting Tribunal tries to vainly to impose standards of programme quality but enforcement is pointless when the receivers are at the door.

There is also a parallel in America with another of the Broadcasting Bill's proposals — to remove certain national sporting events from the protected list and throw them to the open market. Mr David Mellor, the new broadcasting minister at the Home Office, says it is unlikely that owners of the rights to television the Wimbledon tennis championships would with their event to be seen other than by the largest possible home audience. He should look at what has happened in the United States where baseball — the national game — has virtually disappeared from free television and been placed in the hands of the cable barons.

The Broadcasting Bill's proposals are draconian to no purpose. What the Prime Minister dislikes as "the cosy duopoly" of the BBC and ITV has ended anyway with the advance of cable systems and the installations of satellite dishes by the thousand every week. The challenge for public policy now is to protect the excellence of the national services that now exist so that these continue to be available as part of the choice that Mrs Thatcher (hardly a typical consumer of television) wants for viewers.

A good way to get the Prime Minister and the Treasury off the auction hook on which they stuck themselves has been suggested by the Campaign for Quality Television. It is to hold an auction but to specify that the bid represent a promise of money to be spent on programmes, not on sum of money that would be given to the Treasury. Will the voices of reason be heard? As the Broadcasting Bill heads for passage, probably next July, those Tories who fear its potential, should remind the government of another lesson from history — that monuments, once destroyed, cannot be built again. □

Singh shifts India towards nationalism

- Emphasis on technological self-reliance
- Parliament may be split on nuclear policy

New Delhi

THE National Front government led by Vishwanath Pratap Singh, which came to power in India after the November election, intends "substantially" to strengthen research and development and place a strong emphasis on self-reliance in science and technology. Important changes in research priorities and technology import policy are likely.

The National Front is an alliance of five parties that came together before the elections with the main objective of bringing down the Congress government headed by Rajiv Gandhi. Although a minority in the parliament, the front has been assured of support by the right-wing Bharatiya Janata Party and the communists. The approach to science and technology will therefore have to try to reflect the various aspirations of the right, the centre and the left. For the time being Prime Minister Singh, a physicist by training, has himself taken charge of the Ministry of Science and Technology and the Departments of Atomic Energy, Space, Electronics and Ocean Development. A science minister is expected to be inducted into the cabinet later.

The new government professes to be more nationalistic than that of Gandhi and stresses research and development that will make an impact on the quality of life in the villages where 80 per cent of Indians still live. If the government is serious in implementing its election manifesto, a shift from sophisticated to more mundane research is likely. This is consistent with the its decision to divert half of the country's financial resources to the development of rural India. Harnessing non-conventional and renewable sources of energy has been declared as the government's major objective.

The existing liberal import policy may be revised to ensure protection to indigenous technologies which the new government believes is necessary to provide a strong base for self-reliance. According to its manifesto, technology imports will be cut down and work at national laboratories will be directed towards import substitution. Multi-nationals may once again find India a tough market. Singh's cabinet includes a former minister from the post-emergency non-Congress government which banished IBM and Coca Cola from India. The government manifesto blames Gandhi for allocating resources to "indiscriminate automation and thoughtless computerization", which it claims

has displaced labour and increased the country's dependence on foreign capital.

The government's view is that Gandhi wanted to "leapfrog to the twenty-first century", but the new policy is to go "step by step".

On science matters, the front government may be influenced by its ally, the Bharatiya Janata Party, which has 90 members in the Parliament. The party has vowed to use science in the service of the poor and "develop technologies with a human face". It wants to put new life into national laboratories and direct them to develop appropriate technologies for the masses. The party supports measures to stem the brain drain, increase publication

FRENCH RESEARCH

Universities get a little help

Paris

FRENCH education minister Lionel Jospin has announced a new plan to boost university science research. He intends to create a new National University Institute of Advanced Scientific Studies (Institut National des Hautes Etudes Scientifiques de la Recherche Universitaire) to give leading professors as well as young scientists the time and facilities to carry out high-level research.

But no new funds are likely to be set aside for the institute, meaning it will not exist as bricks and mortar. The idea is to create 60 'senior chairs', at a rate of 15 per year, and 30 'junior chairs'. The senior chairs, which will be for ten years, aim to give university scientists "of renown" better facilities within their own departments and some relief from teaching. New lectureships will be created to take over part of their teaching load. In addition, about 20 one-year visiting professorships will be created for foreign scientists.

The plan will help tilt the research balance towards the universities. At present most research is carried out in the *grandes organismes*, notably the CNRS (National Science Research Centre) and INSERM (National Health and Medical Research Institute). Although their laboratories are often near university campuses, researchers do little teaching.

University professors are encouraged to carry out research but unless they are associated (through grants) with CNRS or INSERM, they are not likely to enjoy the same opportunities as full-time researchers.

of scientific literature in regional languages, establish a network of centres for application of science and technology in rural areas and boost research in indigenous systems of medicine.

The National Front may go along with its ally on these matters but on nuclear policy their ideas differ. The National Front would like to continue the existing policy of keeping the options open, but the Bharatiya Janata Party wants "optimum defence preparedness including production of nuclear bombs and delivery systems". It is not clear whether Singh would retain or replace the current science adviser, Professor M. G. K. Menon. Menon had been playing a key role in formulating science plans as a member of the planning commission having been made science adviser, by Gandhi in 1985. He was not available to comment on his role in the new government.

There is no indication whether the scientific advisory committee created by Gandhi will continue or be wound up.

K. S. Jayaraman

The proposal to boost university research comes as education and research are being given high priority by the government. But so far this has been translated only into more money for the CNRS and INSERM, slightly better salaries for university staff and a promise to build new universities.

Peter Coles

Squeeze provokes strike

Paris

THERE are other pressing questions facing the education minister concerning the universities. All over the country, teaching staff, administrators and students are staging walk-outs and protests at working conditions. Most French universities are severely overcrowded and many, built hurriedly in the 1960s and 1970s, are falling apart. This year there were 44,000 more students seeking places than in 1988. In the Paris faculties such as Tolbiac, Jussieu and Creteil, students are forced to stand in the amphitheatres and as many as 60 crowd into seminar-rooms designed for 20, some listening from the corridor.

New universities are being built, but will take several years to complete. In the meantime, the number of candidates is rising each year. All school-leavers who have their baccalaureat are entitled to a place in the first year of university. And the government wants the number of 'bacheliers' to double. To ease the crisis, new annexes are being set up outside Paris in the suburbs and new towns. But school-leavers will still have to queue next July — as every year — in the hope of registering in the faculty of their choice.

Peter Coles

A new direction for HUGO

Washington

WALTER Bodmer, director of research at the Imperial Cancer Research Fund in Britain, was elected last week to replace Victor McKusick of the Johns Hopkins Medical School as president of the Human Genome Organisation (HUGO). The hope is that Bodmer will provide the leadership and political acumen necessary to inject some life into HUGO, which has come under fire for being slow and ineffective.

HUGO was launched a year ago to coordinate international efforts to map and sequence the human genome but has made little progress towards raising the several million dollars a year needed to support its activities. It now has only \$25,000 in the bank. The best prospect for immediate funding appears to be the Howard Hughes Medical Institute, which is considering making a donation of about \$1 million now that HUGO has acquired status as a charitable foundation in Europe and the United States. Initial hopes that HUGO would receive government funds have so far come to nothing and charitable institutions are being looked to for early support.

Bodmer rejects criticisms of HUGO, insisting that there has been "a lot going on behind the scenes" over the past year. While he is president, HUGO's main office will be in London — when the money can be raised to pay for it. The Wellcome Trust is one possible source of support. Other HUGO offices are planned for Washington and Tokyo. Later, there might also be an office in Moscow.

As well as a human genome workshop

to be held in Oxford, HUGO will next year carry out a study of the ethical, legal and social implications of the human genome project under the direction of McKusick. The study will be similar to that being carried out by the US National Institutes of Health (NIH) human genome office but will bring together the views of different countries and different cultures, says Bodmer.

A main aim of HUGO is to encourage international collaboration and the unrestricted exchange of data from the human genome project, an aim somewhat at odds with the views of council member James D. Watson, who heads the NIH

genome office and advocates that data should be kept from countries that do not contribute to the project. Bodmer is quick to point out that Watson is not an active council member and his views do not represent the views of HUGO. Bodmer says that even if restrictions on access to data were desirable, there is no way they could be enforced.

Two new vice-presidents were also elected at HUGO's council meeting last week in Bethesda, Maryland: Charles Cantor, head of the genome programme at Lawrence Berkeley Laboratory and Andre Mirzabekov of the Academy of Sciences of the USSR. Kenichi Matsubara of Osaka University in Japan will serve as the third vice-president for another year.

Christine McGourty

Japan still seeking a role

Tokyo

ON 1 December, Japan's molecular biologists debated their role in the human genome project at the annual meeting of the Molecular Biology Society of Japan in Sendai. It was the first public debate of a major science project by Japanese scientists and comes at a time when Japan has been criticized by Nobel prize winner James Watson for an apparent failure to commit sufficient funds to the international project (*Nature* 342, 463; 1989). The debate revealed the anxieties of some scientists and a growing rift between Japan's leading molecular biologists over which government agency should lead the project.

Kenichi Matsubara, Japan's representative and vice-president of the Human Genome Organization (HUGO), opened the debate by describing the goals of the project and its present status in Japan. Then Michio Oishi, another member of HUGO, voiced some of the concerns of society members. The debate was then opened to the floor.

Michio Oishi, a member of the international HUGO committee, says that MESC officials have given him no clear answer on whether the project will have an effect on other research funds. Oishi stresses that he is a supporter of the project. But it is his personal opinion that the project has many technological aspects and should be led by the Ministry of International Trade and Industry (MITI) or some other technology-oriented agency rather than MESC. He feels that Japan's greatest contribution can be made in the area of robotics and sequencing technology rather than in the area of basic biological research, such as cloning where he says the United States is far ahead.

But Kenichi Matsubara, vice president and Japanese representative of HUGO, says that he is "optimistic" that the human genome project will provide MESC with an opportunity to re-evaluate its system for supporting research and it will not necessarily drain off funds for other subject areas. Matsubara heads a MESC taskforce on the project. He fully recognizes, however, that the project must be an inter-ministry and inter-agency effort. And, earlier this year, he suggested that an "invisible" committee of scientists, like the MESC taskforce, could play a coordinating role (see *Nature* 339, 648; 1989).

The taskforce was recently established by MESC with about ¥600 million (\$4 million) to set up a project over the next two years (*Nature* 340, 667; 1989). Long before this, in the early 1980s, the Science and Technology Agency (STA) began investing about a million dollars a year in the development of automatic DNA sequencing machines in a collaborative programme with industry (*Nature* 325, 771; 1987) and STA is now directing about the same amount into projects at the agency's Institute of Physical and Chemical Research (RIKEN) to sequence a small yeast chromosome and to map and eventually sequence human chromosome 21. STA this year also launched the Genosphere Project under the auspices of its ERATO programme which will provide a few million dollars a year to genome-related research (*Nature* 339, 572; 1989).

The Ministry of Agriculture, Forestry and Fisheries has a tiny project to sequence the rice genome (*Nature* 340, 491; 1989). The Ministry of Health and Welfare has requested about \$2 million for next fiscal year to sequence genes that cause human disease. And other ministries are soon expected to initiate genome projects.

Some of those attending the meeting

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Walter Bodmer: office hunting in Central London?

LOMA PRIETA EARTHQUAKE

Safer structures take priority

were greatly concerned about the recent suggestion by James Watson, director of the US National Institutes of Health Center for Genome Research, that countries failing to contribute financially to the project should be denied access to US genome data, a criticism clearly directed at Japan.

Last month Matsubara replied to Watson's criticisms in *Nature* (*Nature* 342, 463; 1989). But Yoji Ikawa, head of the RIKEN genome project and another HUGO member, says that the Japanese government should issue an official reply to Watson, because he is both a scientist and a representative of the US government. And it is Ikawa's opinion that the Japanese government should first come forward with funds for an international organization that scientists will then run. Others, including Matsubara, think that the scientists themselves must take the initiative.

Behind these arguments lies a battle between scientists and the various government ministries and agencies over who should take the lead in the Human Genome project. Itaru Watanabe, vice-president of the Science Council of Japan, an elected body of 200 academics, says he is greatly concerned about this growing factionalism which he says shows that Japan has no coherent science policy. The Science Council has recently suggested that a new organization should be established to coordinate the human genome research effort. But the council has lost a great deal of its political power since it was re-organized by former Prime Minister Yasuhiro Nakasone.

Younger researchers at the meeting were more concerned about who will actually do the sequencing. As one young scientist said, they are the ones that will probably have to do "the road construction work" and they are worried they will be ordered by their superiors to look at sequences in which they have no interest, a legitimate concern in Japan where professors wield considerable power over their junior staff.

But Oishi and others attending the meeting in Sendai think that much of the sequencing can be carried out by system engineers and companies using new sequencing technology. This is the key philosophy behind the STA project initiated by Professor Akiyoshi Wada in 1981 who believes that sequencing is a job for machines not scientists. Matsubara hopes that MESO will establish new funds for such contract research.

But it is uncertain just how soon such companies will be established in Japan. Matsubara noted that while he has been approached by many US companies since he became vice-president of HUGO, not one Japanese company has come to him to enquire about the project.

David Swinbanks

San Francisco

In the wake of the Loma Prieta earthquake, Congress has already provided an extra \$20 million for seismic research and more seems sure to follow. Now the debate is over how best to spend new funds: early indications are that the emphasis will be on applying existing knowledge to making buildings safer, rather than on rushing to improve the art of earthquake prediction.

Last week, the debate surfaced when scientists, engineers and emergency-response officials testified before a congressional subcommittee that convened at the annual meeting of the American Geophysical Union (AGU) in San Francisco. The mission of the subcommittee on science, research and technology was to help to assess the lessons learned from the 17 October earthquake and plan future funding for the National Earthquake Hazards Reduction Program (NEHRP).

For the most part, testimony was along predictable lines, with seismologists and geophysicists wanting more research on earthquakes and earthquake prediction

other high risk areas as well. Such a project would cost about \$50 million over 10 years in California alone, he estimated.

Taking the other tack was Chris Poland, senior principal of H. J. Degenkolb Associates, Engineers, a San Francisco-based company. Poland argued that there is already a wealth of seismological information that has not been applied in engineering design and that new funds would be better spent in this area. He said this is especially true after the Loma Prieta earthquake, which provided a real-life, strong-motion test of existing theories.

By all yardsticks, NEHRP has received short shrift over the years. The programme was established by a 1977 Act of Congress to advance scientific understanding of earthquakes and drive the practical application of that understanding. Initial funding was \$53 million, equivalent to \$94 million in 1989 dollars. Yet funding for 1989 was just \$66 million, meaning the budget has fallen almost 50 per cent in real terms. No change was expected in 1990. But in the wake of the Loma Prieta earthquake, Congress approved an additional \$20 million, bringing the total appropriated to just over \$87 million.

"All funding for NEHRP has been disappointingly small in comparison to what the original plans were for the programme", said Representative George Brown Jr (Democrat, California), one of the original architects of the programme and a longtime supporter of seismological research. Brown said that he interpreted the field-hearing testimony as indicating that the applications aspect of seismological research had lacked support. Structural research and the development of new building codes and other regulations have not been given as much emphasis as they should, he said.

The principal agencies receiving NEHRP funding are the Federal Emergency Management Agency, the US Geological Survey (USGS), the National Science Foundation (NSF) and the National Institute of Standards and Technology (NIST). Traditionally, the prediction-oriented USGS has dominated the budget, receiving about half of all NEHRP funds. At the other end of the spectrum is NIST, which undertakes a variety of structural research projects. For the past three years NIST has seen its budget frozen at \$525,000 — less than one per cent of the total NEHRP budget.

That percentage is something Congress looks set to change. Of the \$20 million in additional funding approved after Loma Prieta, NIST received \$2 million. NSF, which is also involved in some earthquake engineering research projects, received another \$3 million — the same as the allocation for USGS.

Robert Buderl

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Unsafe structure: a Highway Patrol officer surveys the Oakland Bay bridge soon after the collapse (AP)

and engineers seeking to shore up the structural lines of defence. "We really have not measured enough things with enough instruments", AGU president Don L. Anderson, a geophysicist at the California Institute of Technology, said after the hearing. He said it might be foolish to focus new research moneys on building design and reinforcement, "because the engineers cannot really design a safe structure unless they know what the earthquake is going to do and what the geology is going to do". Anderson testified before the subcommittee in favour of what he called a "research array", a series of up to 100 broadband digital seismometers around California — and eventually in Alaska, Hawaii and

Oldest reptile to leave UK?

London

THE ultimate fate of a unique British fossil hangs in the balance this week following a meeting last Friday of the Reviewing Committee on the Export of Works of Art, acting on behalf of the UK Department of Trade and Industry (DTI). The 338-million-year-old fossil, still not formally named, represents the earliest-known reptile and was described in *Nature* last week (342, 676–678; 1989). The committee heard the case for delaying its sale to a museum in West Germany, but the verdict is still confidential; sources close to the committee expect that it will be announced later this week after consultation with the DTI and the Office of Arts and Libraries. British researchers are hopeful that the committee will recommend that the sale be delayed to give museums in Britain the chance to raise the money to keep the fossil in the UK.

The fossil was discovered in Lower Carboniferous limestones at East Kirkton in Lothian, Scotland, by Edinburgh-based professional collector Stanley Wood, who put the 10-inch specimen on the market at £180,000 exclusive of Value Added Tax. Researchers at the Royal Museum of Scotland and the Natural History Museum in London had expected a more modest sum, somewhere around £40,000, but the price tag exceeds their current budgets. A sale has now been agreed with the Natural History Museum in Stuttgart, where the fossil will find a home in the spring of 1990. Rupert Wild of the Stuttgart Museum was unavailable for comment on Monday.

The sale of any national treasure demands the scrutiny of the British government, who set down the conditions under which an export licence is granted. The committee has never before been required to judge the fate of a fossil, or indeed any other geological specimen. "This is the first time a fossil has become a work of art", said Beverley Halstead of Imperial College, London, one of the independent assessors called upon to advise the committee on the scientific importance of the find.

The committee's deliberations were consequently a new experience for British palaeontologists. In addition to Halstead, Dr Gilbert Larwood of the University of Durham and Professor Alec Panchen of the University of Newcastle upon Tyne advised the panel. Testimony was also submitted by Mr Wood, who was, he says, accorded "a very, very sympathetic hearing". Making the case for delaying the sale were Dr Robin Cocks, Keeper of Palaeontology at the Natural History Museum in London, his colleague Dr Angela Milner, and Dr Ian Rolfe, Keeper of Geology at the National Museums of Scotland.

Less measured, apparently, were the remarks of the committee's chairman, Mr Jonathan Scott, who was, in Halstead's words, "not at all pleased" that details of the matter had found their way into the British press. This broadside may have been directed at Rolfe, who in all innocence had allowed free discussion of the story in the belief that all relevant details — including the price tag — were public knowledge. Milner confirms that this information had been available in the Scottish press as long ago as 1 September, when articles in *The Scotsman* and the *Glasgow Herald* mentioned the possibility of the sale at the price now under discussion. The *Herald* even mentioned the Stuttgart Museum as a potential buyer. But the thing that may have angered Scott most, says Milner, was the presence outside the meeting of one of several press photographers who had hounded Wood on his way to the meeting.

The fossil concerns Rolfe particularly, because the Royal Scottish Museum already houses many fossils collected at East Kirkton. Rolfe, in common with all those involved, was not prepared to comment on the committee's decision at this stage, although it is clear that the committee cannot deny an export licence — the issue is simply whether the sale will be delayed.

DRIFT-NET FISHING

Japan and Taiwan refuse international ban

Sydney

SEVERAL South Pacific nations agreed last month in Wellington, New Zealand, on a ban on drift-net fishing in their territorial seas and exclusive economic and fishing zones. The ban, which emerged from an international convention on the prohibition of fishing with long drift nets, also extends to fishing by any vessels flagged by the signatory countries in a prohibited area designated by the convention. But Japan and Taiwan, two of the biggest participants in what critics say is a destructive and

This decision is based on a judgement of the object's worth according to rules established in 1952 known as the Waverley Criteria, in particular whether the loss of an object would constitute a national "misfortune", whether it has special aesthetic merit, or whether it is of significance to some branch of art, learning or history.

The second and third criteria were not at issue in this particular case. The first criterion was more contentious, because scientific research knows no boundaries; the fossil would, presumably, be as accessible to scholarship in Stuttgart as in Edinburgh, for example.

Wood is highly regarded in the palaeontological community for his almost magical ability to find interesting fossils in unexpected places, but his relationship with museums is sometimes compromised because of his treatment of fossils as objects for sale, not to mention his readiness to talk to the press. On the other hand, museum workers feel that many important fossils would remain undiscovered were it not for Wood's expertise. Wood has now left East Kirkton in search of pastures new — he spoke to *Nature* just before leaving for the Moray Firth, an area well-known for exposures of Devonian age. But he thinks that other fossil reptiles await discovery at East Kirkton: "there's no reason to suspect that there won't be", adds Milner. "There might be dozens more."

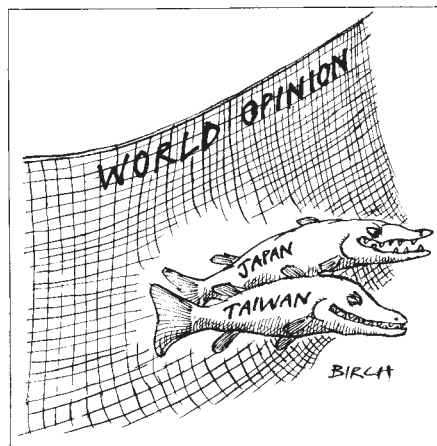
Henry Gee

indiscriminate method of fishing (see *Nature* 342, 9; 1989), dropped out of the negotiations and will not accede to the ban.

The vice-chairperson of the South Pacific conference, Jesse Raglmar of the Federated States of Micronesia, said that the convention would extend the ban over international waters to cover an area from French Polynesia to Australia, bounded by the Marshall Islands to the north. Although the convention has an enforcement clause allowing South Pacific nations to take "appropriate measures" to stop drift-net fishing in their own regions, any such measures attempted outside a country's exclusive economic zone would have to accord with international laws of the sea.

Japan's refusal to participate in the ban was expected. Taiwan withdrew its delegation from the conference after insisting that it should be given government status, despite its not being so recognized by a majority of signatory countries. A spokesperson for the Australian government's Department of Foreign Affairs and Trade said that by trying to secure a statement of support for its longstanding claim to be the real China, "Taiwan appears to be pushing a political objective unrelated to the conference".

Tania Ewing



Berlin retreats in reactor row

Munich

RESEARCHERS in West Berlin breathed a sigh of relief last week as mayor Walter Momper (Social Democrat) gave in to pressure from Bonn and agreed to license quickly a nuclear research reactor at the Hahn-Meitner Institute (HMI). The research committee of the Bundestag had threatened to cut off all funds for new research projects in West Berlin if the licensing of the reactor did not move ahead.

Momper faced a difficult decision as his fragile coalition government includes the Alternative Liste (AL), whose members oppose the operation of all nuclear reactors, including research reactors. At first, he tried to satisfy Bonn's demands by agreeing to license the reactor by May 1990 (see *Nature* **341**, 479; 12 October 1989). But Momper has now been forced to go further and give an assurance to Bonn that all members of the West Berlin Senate, including those from the AL, are in favour of the reactor. Research Minister Heinz Riesenhuber (Christian Democrat) responded last week with DM18.5 million (\$10 million) over five years to the Hahn-Meitner Institute for a new programme in photovoltaics and solar energy.

The Research Ministry (BMFT) paid 90 per cent of the total cost of renovating the reactor — some DM150 million (\$84 million). Riesenhuber and his conservative colleagues in the Bundestag feared that

licensing delays might drag on for so long that the reactor might have to be scrapped without ever having been used. The reactor will serve primarily as a neutron source for physicists, chemists and biologists, mostly from outside HMI.

The West Berlin mayor's change of heart came partly in response to changing political circumstances following the opening of the border with East Germany and the tremendous influx of refugees and visitors. West Berlin now needs more money than ever from the West German government, which already provides more than half of its budget.

But the problems of HMI may not be over yet, according to HMI spokesman Thomas Robertson. Despite Momper's assurances, Environment Senator Michaela Schreyer (AL) may still try to find a way to delay the licensing. One opportunity exists in uncertainty over where waste from the reactor will go.

Plans to dispose of the waste in the United States are affected by a US court decision that prohibits the transport by road of nuclear waste containing highly enriched uranium. HMI is negotiating with Dounreay in Britain for temporary storage rights but it is not certain that Schreyer will accept this alternative.

The sole candidate for the open directorship of HMI, Siegfried Grossmann, has said he will take the job only if the reactor is licensed in May on schedule.

Steven Dickman

ENVIRONMENT

Victory recorded in Mono

San Francisco

IN a sweeping victory for environmentalists, a California judge last week upheld a temporary restraining order forbidding the city of Los Angeles from diverting water from Mono Lake, a unique wildlife habitat in the Sierra Nevada mountains of northern California, and moreover compelling the city to return to the lake some of the water that had already been diverted from its feeder streams into local reservoirs.

The ruling reaffirms earlier legal decisions that the diversion of water from feeder streams threatened the very existence of the alkaline lake, which is a migratory and nesting haven for thousands of rare or endangered birds. A temporary restraining order was granted in June forbidding the Los Angeles Department of Water and Power (LADWP) from removing diverted water from the Mono basin until 31 March. (This date represents the end of the "runoff year", by which time the winter's snow in the mountains has melted.) In August, the LADWP was

ordered to release water from a holding reservoir in the Sierras to raise the lake level to 6377 feet, a foot and a half above its present level.

In September, the LADWP, which receives about 17 per cent of its water from Mono Lake streams, asked the court to reconsider this ruling. But on 6 December Superior Court Judge Terrence M. Finney rejected the appeal, and provided explicit instructions governing the rate of release.

The legal tussle over Mono Lake has become one of California's most contentious environmental issues. Los Angeles has been diverting water from Mono Lake since 1941. In that time the lake level has fallen by 41 feet, and several state and federal government studies have indicated that at the present rate of consumption the lake's ecosystem will collapse early in the next century (see *Nature* **341**, 478; 1989). Environmental groups have rallied to the defence of Mono Lake, whose rich population of algae and brine shrimp attracts many species of birds.

Robert Buderl

Speedy money from Volkswagen

Munich

THE Volkswagen Foundation of West Germany has announced a new programme to aid universities in East Germany. It will create a fund of DM10 million (\$5.6 million) which will be made available in grants to researchers in all fields of natural science, social science and the humanities. A scientific review board to be named by the foundation will assess the applications; the money should begin flowing next year.

The West German government has already announced closer scientific co-operation with East Germany, but the action of the Volkswagen Foundation is the first private initiative of its kind in the aftermath of the collapse of the Berlin Wall. Spokesman Werner Boder said that the foundation's programme, beyond its immediate beneficial effects, is intended to stimulate similar initiatives from others.

The Hannover-based Volkswagen Foundation is the largest private foundation in Europe for the support of science and technology, and in 1988 provided about DM150 million in grants, to research institutions all over the world. Last March, the foundation had already begun to support cooperative projects between researchers in East and West Germany; the first fruit of these efforts will be the publication of a critical edition of *The History of Ancient Art*, written by the historian Johann Joachim Winckelmann in 1764.

Steven Dickman

HUMAN-POWERED FLIGHT

Helicopter hovers for a moment

San Francisco

RELYING on the furious pedalling of Greg McNeil, an engineering student and a member of the US cycling team, students at the California Polytechnic State University in San Luis Obispo have achieved what they called an aeronautical first: a human-powered helicopter. To the cheers of watching students, the ungainly Da Vinci III craft, built mainly from composite materials and plastics, rose at a noticeable tilt several inches into the air before crashing to the ground, breaking one rotor tip.

One purpose of the eight-year project, is to capture the \$25,000 Igor I. Sikorsky Award offered by the American Helicopter Society. But to win the prize, the Da Vinci III will have to stay aloft a full minute and rise three metres in the air.

In the meantime, the students hope to claim an award from the less fussy International Aeronautic Federation. The team is hoping to get official documentation of a human-powered helicopter liftoff, but have not yet been able to confirm the requirement, which they think is a mere 5–10 seconds aloft.

Robert Buderl

IBM rewards new talent

Tokyo

IBM Japan last week announced the 1989 winners of its Japan Science Prize. Unlike Japan's other leading science prizes, the awards go to young (under 45), up-and-coming, Japanese scientists rather than to well-established Western scientists.

Candidates are nominated by university presidents, deans and professors, directors of national research institutes and IBM visiting scientists in the fields of physics, chemistry, computer science and electronics. The prizes are worth ¥3 million (\$20,000) each.

This year's winners are:

- Yoshichika Ohnuki of the University of Tsukuba's Institute of Materials Science, for synthesis of the world's one and only heavy fermion material, CeCu_6 .

- Shunichi Nose of the department of physics at Keio University for development of a constant-temperature molecular dynamics method for computer simulation of thermodynamic problems.

- Masanori Tachiya at the National Chemical Laboratory for Industry in Tsukuba for development of equations to describe reaction dynamics in inhomogeneous systems (for example, a solid material passing into solution).

- Hiroshi Imai, of the department of computer science and communication engineering at Kyushu University for development of efficient algorithms for computational geometric problems, such as mapping and image analysis.

- Masami Hagiya of Kyoto University's Research Institute for Mathematical Sciences for research on the generaliza-

tion of computer programs.

■ Hiroyuki Sakaki of the Research Center for Advanced Science and Technology at the University of Tokyo for research on quantum structure and superlattices in semiconductors, particularly those made of gallium arsenide. Sakaki does not quite fit the image of the prize as he is already an established scientist. His research led to the development of the High Electron Mobility Transistors (HEMT) used, for example, in amplification of electric signals in advanced radio telescopes.

David Swinbanks

UNESCO

No signs of a change of heart

Washington

ALTHOUGH the US State Department is "currently reviewing actions" taken by UNESCO to reform what were seen here as illiberal policies and an overweight bureaucracy (see *Nature* 342, 6; 1989), it was officially made clear last week that there is no likelihood that the United States will rejoin the organization soon. The United States, together with the United Kingdom and Singapore, left UNESCO at the end of 1984. The State Department, which released a statement clarifying its position in response to some recent lobbying and newspaper editorializing in favour of rejoining, said only that the government "hadn't seen anything which would lead us to change our policy."

David Lindley

MEDICAL RESEARCH COUNCIL

Increase softens blows

London

THE UK Medical Research Council (MRC) expresses satisfaction with an increase in support of £82 million over the next three years in its annual report, released this week. But it says that satisfaction has to be "moderated by the knowledge that the increase... still leaves the Council with a budget which will continue to decline in real terms once the effect of the cash injection wears off".

The one-time injection of funds, announced in February, will allow the council to provide backing for the Human Genome Mapping Project (£11 million over three years) and other projects, and to continue its special programmes in AIDS research.

Longer-term planning by the MRC must now wait until January when a decision on the organization of civil research funding is expected from secretary of state John MacGregor. A working party of the Advisory Board to the Research Council

(ABRC) has already examined the Morris report, which proposed that Britain's five research councils should be replaced by a single National Research Council, and submitted its confidential advice to the secretary of state.

The MRC report reiterates its opposition to the Morris proposals, describing them as "damaging to the interests of medical research". Under the Morris proposals, the MRC would remain largely unaltered, as a medical division of the NRC. But what worries the MRC is the possibility that some responsibilities for basic biological research might be transferred to a new biology and environment division of the NRC, meaning that clinical medicine and basic research would be separated. Concern over overlaps in the research councils' responsibility for biological research was a key factor driving the Morris report proposals (see *Nature* 339, 165; 1989).

Peter Aldhous

Crisis . . . what crisis?

Washington

THE US National Commission on AIDS last week harshly criticized US efforts to tackle the AIDS epidemic in its first report to President George Bush. The commission says that a "dangerous" complacency towards the epidemic is developing and calls for major changes in the nation's system of health care.

The commission, an independent body set up in August by Congress to advise Congress and the president on policy issues relevant to the AIDS epidemic, includes members of the Bush administration as well as experts in health care. Its first report was not due until August 1990, but after interviewing researchers, physicians and public health officials over the past three months, the commission decided that the problems were too urgent to wait until then.

It lays the blame for inadequate health care services on the federal government, which it says has no plan for coping with the epidemic. In the federal government's widely publicized drug control strategy, it fails to acknowledge the link between drug use and HIV (human immunodeficiency virus) infection and in 90 pages of text it mentions AIDS only four times; in the recommendation and discussions on allocation of resources it does not mention AIDS at all.

The commission says that it is estimated that by 1991, AIDS will be among the top ten leading causes of death in the United States and, for people between the ages of 25 and 44, deaths from AIDS will far exceed those from all other causes.

The main problem for many AIDS patients is the difficulty in obtaining health care under Medicaid, the financial reimbursement programme which caters for people with low incomes. Obstacles to obtaining care under the Medicaid system are often insurmountable, says the commission, and any belief that this system is adequate for all those who need it is a "fantasy" according to one witness consulted by the commission. Also contributing to the problems are the "gross lack of training" for doctors to treat people with HIV infection and AIDS, and the "serious disincentives" for doctors to treat poor people.

At the top of its list of requests is that there should be a frank recognition that a crisis exists, accompanied by a clear definition of the responsibilities within government for tackling the problem. The commission also calls for a network of regional centres to care for patients as well as for special units to treat drug addicts who are HIV-infected.

Christine McGourty

UK university rankings

SIR—W. I. Montgomery has recently proposed (*Nature* **341**, 562; 1989) that there is a negative correlation between the research grades allocated to universities by the Universities Funding Council (UFC) and the teaching quality as inferred from the percentage of graduates unemployed or in short-term work. The latter statistics were published by the *Financial Times* (*FT*) on 13 September 1989. There are a number of weaknesses in this analysis.

First, it is proposed to omit, on the one hand, Oxford and Cambridge, "which are exceptional in terms of research performance and whose graduates are moderately successful in avoiding the dole queues", and on the other hand Ulster and Keele "where research and teaching both fare badly using the present criteria". I submit that the proposed omission without good reason of four out of 45 universities because they are patent outliers for the hypothesis being tested is playing skittles with statistics.

Second, some universities or colleges are included in one table but not in the other. Montgomery does not explain fully what he has done with these cases.

Third, substantial number of graduates (3.1–15.7 per cent per institution) could not be traced in the *FT* survey and students who read for degrees for personal interest and satisfaction rather than with the intention of using the qualification to obtain employment are included in the unemployed. The *FT* questions whether students should be accepted by universities if they do not intend to work after graduation. That is another problem, but the method of presentation of the survey by the *FT* probably gives a distorted reflection of the views held by potential employers concerning the quality of graduates of a particular university.

Fourth, some institutions (for example, Brunel, Salford and Aston) are mainly geared to teaching and research in pure and especially applied sciences. Not surprisingly, the percentage of students of these institutions who find employment quickly is greater than in the case of universities offering a broader range of academic disciplines. While bank interest rates remain high, it is understandable if employers tend to focus their attention more sharply on graduates with the most immediately pertinent qualification rather than possibly better graduates in less relevant disciplines.

Finally, students in Northern Ireland in particular, but also in parts of Scotland and Wales, tend to want to stay in a familiar environment in preference to migrating to those parts of the United Kingdom where unemployment is lower and graduates are in greater demand. This may partially explain the low position of

Ulster, St Andrews, Edinburgh and Wales in the *FT* graduate employment survey.

The other variable in the analysis, the UFC research grading, is also not free from criticism. The grading allocated to a university department is partly a function of external research money acquired. It is well known that the research councils have been able to fund only a small percentage of alphanumerated research applications. The remainder, whether rated alpha, beta or rejected as unsound, do not affect the UFC grading, to the best of my knowledge. In other words, there are good research ideas emanating from many institutions that are not funded because of shortage of money and one may conclude that UFC gradings are influenced to some extent by government policy towards funding of scientific research in universities.

In summary, an attempt to establish a correlation between two parameters, one of which is seriously flawed quantitatively while the other is influenced by economic and political factors in addition to academic factors, is not a fruitful exercise. Montgomery's last sentence is an open invitation to government, should this be felt necessary, to downgrade some departments or even universities unjustifiably to a teaching role only.

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Sri Lankan death

SIR—We were saddened to receive the news of the death of a close colleague, Dr Rajani Thiranagama of the University of Jaffna, Sri Lanka. She was shot dead by an unidentified gunman on 21 September when returning to her house after teaching in the Medical School where she was head of the department of anatomy.

Thiranagama was a prominent member of University Teachers for Human Rights (UTHR), a monitoring organization based at the Universities of Colombo and Jaffna. She had been active in compiling and circulating several reports documenting human rights violations by militant opposition groups, as well as the Indian armed forces who control the north and east of Sri Lanka. She had been harassed and threatened repeatedly since the contents of these reports, which included evidence of killings, became known.

Thiranagama has close links with the University of Liverpool, where she obtained her PhD in 1986. She paid

Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only. □

annual study visits to our department, and was highly regarded by all who knew her. Rajani's friends and colleagues in Britain are writing letters to the Sri Lankan and Indian authorities, appealing for an independent inquiry into the circumstances surrounding her death. We hope that individuals and institutions in other countries will add their weight to our appeal, especially since other Sri Lankan academics who are members of UTHR are now clearly at risk.

Letters may be sent to the Vice-Chancellor, University of Jaffna, Thirunelvely, Jaffna, Sri Lanka, who will forward them to the appropriate authorities. Further information about the campaign may be obtained from A.T.C., who may be contacted by electronic mail on MJ02 @ UK.AC.LIVERPOOL.

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Journal reviewers

SIR—We disagree with David R. Hershey (*Nature* **340**, 424; 1989), who suggests that reviewers have no incentive to do an excellent job because they receive no pay, no recognition and no blame for a poor review. We believe that the satisfaction of participating in this important and traditional process is a significant incentive. We agree with Hershey that further incentives are justified.

Journal reviewers unselfishly donate their professional time to assist an editor in the decision whether a manuscript should be accepted, revised or rejected. The task of reviewing a paper may take several days. As such, consideration should be given to remunerating reviewers. Who, however, can afford to reimburse a reviewer even for a fraction of the time spent in this important work?

Editors should continue to publish the names of their reviewers in the journal. We suggest that journal editors should also write a letter of thanks to a reviewer together with a brief note about the quality of the review. Reviewers may then submit the letter along with their curriculum vitae to their department head when decisions concerning academic appointments, advancement and salary are to be made. Department heads should regard participation as a journal reviewer as an important aspect of an academician's curriculum vitae.

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What price a carbon tax?

SIR—The proposition of a “carbon tax on fuels proportional to the carbon dioxide they emit”, put forward by former Environment Minister Nicholas Ridley, is echoed in the Department of Environment’s Pearce report as a means of putting a price on environmental benefits and losses¹. But can a carbon tax be a realistic and cost-effective way of combating greenhouse global warming?

Methane has 30 times the greenhouse potential of CO₂, molecule for molecule. And leakages of methane associated with natural gas production and distribution are significant, being estimated at 3–6 per cent in the United States (D. Abrahamson, unpublished data) and 3–10 per cent (by me) for the British North Sea gas. This means that, despite the lower emission of CO₂ per unit of heat in gas combustion, the gross contribution of gas for the greenhouse effect is no better than that of coal or oil.

Second, the major chlorofluorocarbon gases (CFC-10 and CFC-11) have some 10,000 times the greenhouse potential of CO₂. A tax of say 5 pence per litre on petrol would scale to £500 per litre on CFCs. Could the economists stomach such a level of tax, even if there were rebates for essential medical uses?

Yet even that tax would be too low to produce substantial cuts in fuel consumption, while the differential tax between gas and coal, amounting to only 2 pence of the 5 pence, would not induce the fuel switching that Pearce *et al.*¹ want. The Association for Conservation of Energy³ “believes current energy prices would have to double if there is to be any real impact on consumer behaviour”.

But supposing that the tax were 5 pence per litre of oil equivalent; its yield in Britain would be £45,000 million a year in Britain if it were levied on coal, gas and oil in proportion to the CO₂ emission (600 Mt per yr²). That is a significant sum for the British Treasury. What would happen if only 10 per cent of it were invested in energy efficiency and conservation measures?

Dr Tim Jackson’s evidence to the Hinkley Point “C” PWR Inquiry⁴ gives figures roughly in the range £5–30 per tonne CO₂ saved for various current technologies, including combined heat-and-power, renewable resources, domestic and office heat insulation, energy-efficient lighting and so on, taking into account their various lifetimes and discount rates of 8–10 per cent per yr. At say 15 per tonne, the £4,500 million would save 300 Mt or half our total CO₂ emissions. In comparison, the £45,000 million tax increase might directly effect 60 Mt savings.

While the Treasury would no doubt welcome a carbon tax windfall, it is clear

that direct investment in energy-saving technologies is far more effective (50 times, on my figures) than taxation.

In practice, in a market economy, the way forward is through tax allowances and energy-efficiency programmes (the current puny level of under £20 million a year is even being cut back under present policy), as the House of Commons’ Energy Committee advocated⁴. Whether such fiscal and promotional measures are financed by a normal carbon tax at say 0.5 pence per litre oil equivalent (plus £50 per litre on CFCs) or by general taxation is immaterial.

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1. Pearce, D. *et al.* *The Implications of Sustainable Development for Resource Accounting, Project Appraisal and Integrated Environmental Policy*, London Environmental Centre, 1989.
2. *World Climate Change Report No. 0*, 24 (Bureau of National Affairs Inc., Washington DC, 1989).
3. *Energy Policy Implications of the Greenhouse Effect Vol. 1* (Energy Committee 6th Report, House of Commons, 1989).
4. Jackson, T. *FoE-10*, Hinkley Inquiry secretariat, Cannington, Somerset, June 1989.

What happened?

SIR—Members of the growing band of biomedical scientists who are becoming disenchanted with the current science funding situation should recall the words of Dr F. W. Twort: “I regret that financial considerations have prevented my carrying these researches to a definite conclusion, but I have indicated the lines along which others more fortunately situated can proceed.” (Twort, F. W. ‘An investigation in the nature of ultra-microscopic viruses’, *Lancet* ii: 1241–1243; 1915).

Twort is one of the people after whom was named the Twort-d’Herelle phenomenon which became known as bacteriophage, the study of which helped greatly to spawn molecular biology; the burgeoning study of this subject is one reason there are not enough funds to go around.

Perhaps some reader can tell us what happened to Twort. Was he assigned a bigger teaching load? Did he become an administrator? Did he later drive a cab in London?

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Carbon dating

SIR—With respect to your News item on radiocarbon laboratories (*Nature* 341, 267; 1989), we wish to correct some factual inaccuracies and to emphasize that the recent carbon-14 workshop marked a significant step forward by the international

dating community towards improving and refining an already high degree of analytical care, accuracy and precision.

The meeting in East Kilbride reviewed the results of an international (not only “British”) study in which around 40 laboratories worldwide analysed a suite of samples of varying type and age. Rather than finding “a disturbing pattern of errors”, the comparison found evidence of currently unexplained variation in the results, which of course explains why the carbon-14 community is undertaking a continuing programme to ensure the quality of its results. In fact, given that a carbon-14 dating analysis requires assay of 1 carbon-14 atom per 10¹² to 10¹⁴ carbon atoms, the level of agreement between laboratories is remarkable. Further to refine the accuracy and precision of dating, however, the meeting agreed a programme of improvements, including (1) the distribution of new reference materials by the International Atomic Energy Agency, (2) more regular in-house analysis of reference materials and (3) continued ‘blind’ testing programmes organized by ourselves.

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Hidden increases

SIR—*Nature* has quite often looked at the costs of scientific periodicals (for example 341, 349; 1989), but I am not sure it has considered the dubious practice publishers sometimes have of adding to periodicals new sections that subscribers must take whether they want them or not. Publicity often tries to make us think we are getting something extra for nothing when in fact there are usually above-average price increases that are not mentioned. For example, *Tetrahedron* is to increase in price by £400 (30 per cent) to accommodate *Tetrahedron: Asymmetry*, and *Journal of Materials Science* will spawn new periodicals on materials in electronics and medicine at an extra cost of £200 (again nearly 30 per cent). My budget usually declines in real terms so that, as in many libraries, coping with even average price rises often requires the cancellation of some titles to pay for the remainder. Huge increases like these, often at short notice, do not help in budgetary planning or endear periodical publishers to librarians.

IAN WINSHIP

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Keeping up with the Russians

George W. Fisher, Priscilla C. Grew and Bruce Yardley

The United States and Britain have a lot to learn from the Soviet way of doing science — not least from the system of allocating research funds.

How often do papers in Western journals refer to the work of Soviet scientists? Rarely at best. Many Westerners dismiss Soviet science as old-fashioned and mired in the bog of a ruined economy.

Yet most specialists can name a Soviet scientist or two in their fields whose creative work has led the subject into new directions. In the Earth sciences, for example, D. S. Korzhinskii and colleagues used thermodynamic theory to interpret ore deposition and other geochemical processes decades before Western geologists caught on. V. I. Vernadskii recognized the biological component of geochemical processes and the role of radioactive heat in geophysics long before his counterparts in the West. Stishovite, the important high-pressure silica polymorph was discovered in a Soviet experiment in 1961. Are Korzhinskii and Vernadskii at work somewhere in the Soviet Union today, unrecognized by Western scientists? Is there something inherent in the Soviet style of science that fosters such unexpected advances? If so, the new spirit of *glasnost* may allow us to benefit from ideas from the East.

Recently, when in Moscow for the Symposium on Physico-Chemical Methods in Petrology, held in honour of the ninetieth anniversary of Korzhinskii's birth, we were invited to visit the Institute of Experimental Mineralogy (IEM) at Chernogolovka, a formerly restricted scientific centre about 40 kilometres north-east of Moscow. Our visit, made possible by the director of IEM, Vilen A. Zharikov, was particularly poignant as Korzhinskii founded the institute in 1969 and, as Zharikov remarked, still serves as "its inspiration and its conscience". We were able to inspect equipment, admire posters and family snapshots, and talk candidly about science and shopping, teenagers and thermocouples, spectrometers and conscience.

Research

At IEM we found a world-class experimental facility and saw innovative research being done that would be very difficult to finance in the West. The Soviet and Western styles of supporting science are very different. In some respects, the Soviet system is effective and flexible. As the West rethinks its funding practices in this era of serious budget constraints, there are important lessons to be learnt from the Soviet Union.

The institute has a staff of about 300 people, of whom about 70 are research scientists. Most of the rest are technical support staff who build, maintain and operate scientific equipment on a day-to-day basis. The size of the technical staff reflects a major departure from Western European, Japanese and especially North American practice, where researchers and their students fill the roles of technicians to a much greater extent.

The institute is divided into nine 'laboratories', each administered by its own head and a chief scientist. These units carry out research on magmatism, metamorphism, hydrothermal processes, metasomatism, gem mineralogy, ore-mineral equilibria, mantle processes, physical-chemical methods and mathematical methods. The last two groups also provide support services for the entire institute. The physical-chemical methods group has modern equipment, including an automated electron microprobe, a scanning electron microscope, and apparatus for infrared and laser Raman spectroscopy, Mössbauer studies and X-ray diffraction. The mathematical group operates a central computer in the institute and has access to more powerful external computing facilities. An engineering branch produces the experimental apparatus for the institute, whereas most of the electronic equipment is purchased externally. An additional, centralized 'collective farm' makes available more general equipment, including hydrothermal autoclaves, internally heated gas apparatus and piston-cylinder apparatus.

We toured many of the experimental laboratories, including some heavily shielded against potential accidents with high-pressure equipment, and we even saw the room with the photocopier (likewise highly fortified!). The quality and abundance of research equipment at the institute was both surprising and impressive. Most of the apparatus for phase-equilibrium studies at high temperature and pressure had been built in-house, and appeared to be of high quality. In addition to conventional experimental equipment, we saw a high-pressure piston-cylinder device of promising new design, and a diamond anvil mounted on an X-ray diffractometer for work at very high pressures. We were told that access to computers had posed a real problem, but that the institute was making good progress on central computer facilities, and

saw numerous personal computers, including several IBM-compatible machines. Discussions with Earth scientists from elsewhere in the Soviet Union showed that such equipment is by no means universally available, but it is clear that at least some Soviet institutes are as well equipped as their Western counterparts.

A notable difference from scientific life in the West is the clear separation of research and education. There is only limited mobility between research posts and university appointments, which is not to say that no education goes on at IEM — several scientists there have part-time faculty posts at Moscow State University, and about a dozen graduate and post-graduate students do research at the institute. Other young scientists begin their careers guided by senior institute mentors. Korzhinskii himself was celebrated for his nurturing of young scientists, and many students who worked with him staff the main petrological institutes today.

Funding

Most of the institute's financial support, about 2 million roubles (\$3.2 million) annually, comes directly from the Academy of Sciences, and amounts to a block grant which is spent on salaries, operations and core research. An additional 1 million roubles (\$1.6 million) is earmarked for programmes covering specific areas of fundamental research that are considered timely, but need not have any immediate economic relevance (somewhat similar to 'special topic' funding in Britain). A current research programme receiving this special funding, initiated by IEM staff, is the topic of 'metamorphism and cratonization'.

Research is planned on a five-year basis, largely by *ad hoc* teams of institute scientists who wish to collaborate on a particular project. These research teams may be led by any scientist, regardless of rank in the institute; some are drawn entirely from within an individual laboratory, others cross laboratory boundaries. Each team draws up its research plans and submits them to the institute's scientific council. The council, composed of all the institute's scientists, allocates support for each project, rules on new appointments, and serves as a colloquium in which papers are presented before publication.

This system of support means that there is less pressure for immediate publication than in the West, and less national peer

control over research projects. Many Western scientists would expect that, without competitive pressures, individual scientists might be less active and less productive, and they would argue that much pedestrian research in certain fields is a by-product of the Soviet system. Even though Soviet scientists work under different pressures, however, the best of them are just as productive and hard-working as their Western counterparts. And there is another side to this story. We saw several innovative projects that would almost certainly fail to survive the peer-review process in the United States or Britain. Until recently, the success rate for grant applications in the Earth sciences in these two countries was such that innovative proposals stood a reasonable chance of being supported. But during the 1980s, increased competition for a diminishing budget has meant that proposals need almost unanimous approval from reviewers. In the process of weeding out pedestrian projects, the system eliminates almost all of the really innovative projects, which are often too controversial to generate universal approval. Most proposals that attract funding are those which take just one small step down a path that is currently recognized by the community as 'opportunistic', 'important' or 'timely'. Zharikov stressed to us that Korzhinskii's career was marked by 'novelty, innovation and controversy' — would his research have met Western market tests?

One of the imaginative projects we saw involved permeability experiments. Many geochemical processes result from interaction between a rock and a fluid phase percolating through that rock. Common examples are the reactions which control the composition of metamorphic rocks, create ore deposits and disperse radioactive elements from waste repositories. The dynamics of these processes depend largely on the porosity and permeability of the rock in question. Although we know quite a bit about permeability in such porous media as sandstones, we know almost nothing about the permeability of deep-seated rocks *in situ*. Scientists at IEM are currently conducting percolation experiments on various natural rocks to determine their permeability under a range of crustal temperatures and pressures.

Any Western petrologist could think of a dozen potential problems with these experiments. It is very hard, for example, to be certain that the geometry of the channels along grain boundaries in a rock sample collected at the Earth's surface is really representative of the form of those channels when the rock was deeply buried, and undergoing prolonged annealing under ambient temperature and pressure. Several factors — differential expansion of mineral grains during unroofing by erosion, a variety of weathering

reactions, or even the strains induced by sample collection and preparation — could alter the form of the minute passageways. Uncertainties such as these would almost certainly doom a proposal to carry out experiments of this sort in the West. Supporters of the Western funding system would doubtless argue that the data from these experiments would be subject to such large uncertainties as to be worthless, and there is some truth to that claim. But at the same time, even poorly controlled experiments on poorly understood problems usually reveal something about the problem, even if only to indicate

In the United States and Britain, the grant system eliminates almost all of the really innovative projects, which are often too controversial to generate universal approval. Most proposals that attract funding are those which take just one small step down a path that is currently recognized by the community as 'opportunistic', 'important' or 'timely'. Zharikov stressed to us that Korzhinskii's career was marked by 'novelty, innovation and controversy' — would his research have met Western market tests?

how the experiment should be done the next time. And if these imperfectly controlled experiments on the frontier are not performed, we may never learn how to design better experiments in the future.

Overall, we have the impression that, consciously or not, the Soviet system tolerates some routine work in hopes of nurturing a new and unexpected discovery that will open up a totally new view of a subject. In the West, on the other hand, we are so preoccupied with ensuring that no funds are wasted on unpredictable or apparently pedestrian research that we often fail to support truly innovative work.

We treasure the freedom and mobility of Western science, the close integration of teaching and research, and the comforts of a more efficient economic system. But Soviet science has its good side. The Soviet system of supporting institutions with strong track records in research and distributing those funds internally, rather than awarding grants directly to individual investigators, allows scientists to concentrate on research instead of the proposal mill. Young scientists can more easily obtain support for that crucial first research project. The five-year time span associated with many research projects permits exploration of new areas with little concern for an immediate pay-off. The relative prestige of science attracts many excellent students to the profession. Soviet scientists are not under the same

pressure to publish early and often, so giving them more time in the laboratory to pursue longer term, more fundamental research, for which they can develop new techniques or learn skills from another field without affecting their promotion prospects or chances of future grants. The Soviet willingness to provide technical help means that researchers can devote their time to research, with fewer hours spent tinkering with unreliable instruments or on routine equipment maintenance.

The difficulties that currently beset the funding of science in the West have crept up gradually on a system which was never designed to operate under such severe conditions. We are wary of drawing simplistic lessons from the Soviet system and applying them to the United States and Britain alike, if only because there are significant differences in the way research is funded in the two Western countries. Merely changing the system by which money is allocated cannot be a substitute for an adequate level of support. But as politicians and agency directors review Western funding systems, they should be prepared to learn from the strengths of the Soviet system, particularly in the promotion of originality.

Of course, the Soviet way of supporting science has its risks — inefficiency, favouritism and stagnation to name but three. But so does the Western system — brain-drains to more lucrative professions, pursuit of the predictable and preoccupation with the bottom line. Perhaps we still need to keep up with the Russians after all. This would involve making science prestigious enough to attract the best students, giving out some five-year research grants, and allocating a percentage of block funding to research institutions (perhaps as an expanded version of the US National Institutes of Health biomedical research grants programme) so that scientists can try projects too novel to compete in a marketplace where only consensual research gets grants.

At a time when the political map of Europe is changing almost daily, instead of complaining about laboratories hidden behind an iron curtain we should try to respond constructively to scientists striving to be active members of the world community. Rather than dismissing Soviet science as backward, we must be prepared to admit that superficially slower science can sometimes produce results without price. □

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Is the salami sliced too thinly?

Every working scientist agrees that there is too much to read, yet most of those who make the complaint are collectively also those responsible for the common difficulty — that the literature of research is overblown.

THE origins of the simile of the scientific literature as a collection of salami slices is anybody's guess, but it is compelling. The image is a representation of the whole body of discovery as a long thin sausage, and of the items of discovery that see their way into print as the slices into which the sausage may be sliced with a sufficiently sharp knife. Of course, there are several philosophical objections; the path of discovery is not linear, for example, while many items of discovery lead nowhere, so that the corresponding salami slices may be considered to have been added in eccentric ways. But the simile has the virtue of suggesting that the process of discovery is a continuous process. Phrases such as "If I have seen further than other men, it is because I have stood on the shoulders of giants" or "One thing leads to another" apply.

The imagery is also capable of misinterpretation. For one thing, the general shape and size of the sausage can be recognized only retrospectively, after all the pieces have been added. But the most serious difficulty is that a slice of salami, like a piece of string, can be of arbitrary size. Much depends on the cutting edge of the knife and the intentions of the one who wields it. That is why the process of publication itself has been likened to that of slicing a salami. And there is a general suspicion that, as things are, the slices are too thin.

Can that be true? And how, for that matter, is it possible, at the leading edge of a growing sausage, to tell what is the appropriate thickness of a slice? Naturally, there can be no simple rule. Defining what physicists would call a lower bound could, in any case, quickly be falsified. The literature is liberally sprinkled with contributions that amount to no more than records of isolated observations — a Voyager photograph of the surface of Jupiter's moon Io, for example, or the first few records of living things at the bottoms of the deep oceans. The first account of the discovery of a pulsating star (at that stage innocent of an explanation) was similarly an intrinsically thin slice. If there should be, say, a single well-authenticated case of water running uphill, that would be a scientific observation well worthy of publication.

That is why the estimation of what constitutes a proper slice must necessarily be subjective. But even then, there are snags. It may, for example, be held that,

to qualify for publication, an intended contribution to the scientific literature should be meaningful, which begs an obvious question: meaningful to whom? To close colleagues and/or competitors? To others in related fields who may profit from or be dependent on the results? Or to science more generally?

In principle, that string of questions (which may be elaborated in obvious ways) should at least be the starting point for a classification of what gets published in journals of different kinds. So far as general journals like this are concerned, for example, there is no point in the publication of material likely to interest only colleagues and competitors. (In passing, it is crucial that this does not constitute a bar to the publication of technical developments in journals such as *Nature*, especially as now when the adaptation of techniques developed in one field to use in another is so fruitful.) In an ideal world, what is published as original research in a journal such as this should be of more general interest than to practitioners in the single field concerned, but even that criterion does not help to define the proper salami slice.

Completeness does. These days, when the vigilance of authors on behalf of their publications seems to be matched by the vigilance of the referees for the quality of what is published, the chances that unsubstantiated observations will find their way into print diminish with the passage of time. If referees err consistently, they do so in their demands for extra information, often requiring that further experiments should be carried out. (People's failure to comply is a principal cause of casualty among the manuscripts submitted for publication.) But referees are less vigilant about completeness, and why should they be otherwise? At the best of times, they do a thankless job for nothing; why should they assess not just what an author has done, but what he might have done?

None of this implies that even the criterion of completeness is exact, although it is more satisfactory than the question about the length of a piece of string. It is almost a literary criterion. Just as it is a poor essay that does not end by answering the arresting question with which it begins, so it is much less than satisfactory that a research article should begin by listing a long string of important questions and then finish by answering

only the least important of them. In that sense, most contributions to the scientific literature embody their own definitions of completeness — which should be taken more literally.

If it were indeed possible to apply this criterion rigorously, there seems very little doubt that the scientific literature would be more compact and more manageable. Sadly, experience shows that it is not so easy. For some purposes, for example, it may be sufficient to clone a gene, but for others nothing less than the nucleotide sequence will suffice. Who is to say that the first step towards the second is intrinsically unremarkable? And what is to be said of a careful demonstration that the chances that Cygnus-X3 is a source of gamma rays have been reduced by a further order of magnitude?

The suspicion that salami-slicing is rife has other origins, chiefly the common knowledge of the incentives that people have for behaving in this way. So long as there is a sense in which the volume of publications counts for important things — the award of research grants, promotion (for tenured people) or job security (for those on short-term contracts), the temptations must be great. Not even the bad habit of relying on citation indices will solve the problem. Indeed, it would be an interesting exercise to discover whether, when people have published two essentially similar but not identical research articles, they have succeeded somehow in doubling their citation record.

The conventional remedy for this state of affairs is that journals should be more vigilant, but there are serious limits to what journals can accomplish on their own. The ideal would be that academic institutions should find more subtle and just ways of assessing the merits of those who belong to them except by some measurement of the volume of publications, but the chances of that coming about are small. Perhaps the most urgent need is to find some means of subverting the cruel rule that, in discovery, no purpose is served in being second with even a tiny gobblet of discovery. Quite apart from its injustice, that is the greatest single incentive to premature publication. But journals which try to persuade authors of similar discoveries to lump their research reports together would not get very far.

John Maddox

Limbs: a pattern emerges

Julian Lewis and Paul Martin

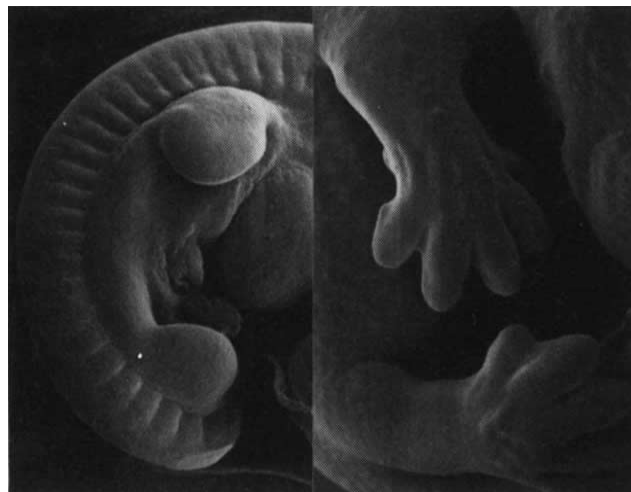
EACH segment of a vertebrate limb is constructed from the same set of cell types — cartilage, muscle, tendon and so forth. The segments differ in that these cell types are arranged in different spatial patterns. But what causes the patterns to be different? Several decades of microsurgical experimentation have yielded the abstract outlines of an answer. The cells in different regions of the early embryo, though they may be destined to give rise to the same range of differentiated cell types, are nonetheless thought to be non-equivalent: they are somehow stamped with different 'positional values' that regulate the subsequent patterning process. Despite their importance in embryological thinking, positional values, like genes before DNA, have long been an abstraction in search of molecular embodiment. Findings reported on page 767 of this issue¹, however, indicate that a large part of the molecular basis of positional value in the vertebrate limb is coming into view at last, and that it resides in the activation of a homoeotic selector gene complex — the same genetic system that serves a similar role elsewhere, marking out differences between segments along the central axis of the body, both in vertebrates and in arthropods.

As discussed by one of us in a recent News and Views article², homoeotic selector (HOM) gene complexes were first discovered in *Drosophila*, as loci of mutations that transform one body segment (or group of segments) homoeotically into another. Each HOM complex consists of a string of related genes coding for transcription factors, all containing a homoeobox sequence. The genes in the complex are brought into play according to a remarkable rule first noted in the fly: with minor qualifications, the further a gene lies from the beginning of the HOM complex in the chromosome, the more posterior its expression domain along the body axis.

Mammals have at least four HOM gene complexes homologous to those of insects. At least two of these complexes, called *Hox-2* and *Hox-5* in the mouse, are known to be expressed along the central body axis according to the same rule as in insects, so that the chromosomal extent of *Hox-2* and/or *Hox-5* activation provides cells with a yardstick for their cranio-caudal position^{3,4}. Although there is still no direct proof that these gene complexes actually regulate pattern for-

mation, studies in *Xenopus* provide strong indications that they do. An anterior excess of the product of the homoeobox gene *Xhox3* results in a failure of development of head structures⁵, and an injection of antibodies against the product of another homoeobox gene, *XIHbox1* (probably a HOM gene corresponding to mouse *Hox-6.1* — see ref. 6), causes a homoeotic transformation of anterior spinal cord into a structure resembling hindbrain⁷.

Several previous papers have reported expression of homoeobox genes in vertebrate limb buds^{6,8-10}. But because such genes are known to have many disparate



Limb development in a mouse embryo: 10.5 days of gestation on the left, 14 days on the right. The same genes that control the patterning of the central body axis may also control limb patterning.

functions, it is not clear what these findings might signify. Dollé *et al.*¹ have now cloned five contiguous members of the *Hox-5* gene complex in the mouse and have mapped their patterns of expression in limb buds by *in situ* hybridization, at six different stages of development. The result is a huge mass of data, which is all the harder to digest because the three-dimensional pictures have to be reconstructed from serial sections and it is often uncertain precisely how the plane of sectioning is related to the natural axes of the limb buds. But there are several general features that stand out clearly and independently of any uncertainties about the geometry of the limb sections. All five genes are expressed in the limb buds. Each gene has a characteristic spatial domain and time of activation. The spatial domains of activation of the genes at any instant are nested, like a set of Russian dolls, with the order of nesting corresponding to the ordering of the genes in the chromosome. The extremity of this nested arrangement, where the largest number of genes is expressed, lies

in the posterior part of the far end of the limb bud; this is the high point of a spatial gradient of *Hox-5* activation.

The spatial pattern appears to be the reflection of a temporal rule: cells in the posterior distal region activate their *Hox-5* genes progressively in chromosomal order; as the bud grows, cells become displaced away from this region and halt any further activation of their *Hox-5* genes. At later stages, expression of the genes is down-regulated in specific parts of their initial domains (for example, in the regions of joints), and in a way that suggests repression of early-activated genes by the products of late-activated genes.

All this parallels the pattern of expression of HOM genes elsewhere, in insects as well as mammals. It implies that the extent of activation of the *Hox-5* complex

may serve to define one dimension of positional value in the vertebrate limb bud (though Dollé *et al.* try to squeeze a second dimension out of their data, based on gradations in the concentrations of the individual gene products).

The challenge now is to relate these findings to the other knowledge that we have about how limbs develop. From grafting experiments, done mainly in chick embryos, it has been argued that two different mechanisms serve to define, respectively, the proximo-distal (base-to-tip) and antero-posterior (thumb-to-little-finger) dimensions of positional value. The first depends on an influence from the apical ectodermal ridge at the distal end of the limb bud¹¹: this keeps the distal mesenchyme just beneath it, in the so-called progress zone, in a labile state. Here the cells display an autonomous tendency to become progressively more and more distal in their proximo-distal positional value as time goes by. As the bud grows, successive cohorts of cells become displaced out of the progress zone and their positional values thereupon become frozen. By this time-dependent mechanism, a spatial gradient of positional values is set up along the proximo-distal axis.

The antero-posterior component of positional value, by contrast, is thought to be determined by the concentration of a chemical signal, or morphogen, emitted by the cells of the polarizing zone (or ZPA) at the distal posterior margin of the bud¹². The signal affects cells in the distal labile region¹³: if they lie close to the polarizing zone and thus receive a high dose of morphogen, they form structures of a posterior type (such as little finger); if they lie far from the polarizing zone and receive a low dose, they form anter-

ior structures (such as thumb). Thus polarizing tissue grafted into the anterior margin of a limb bud will cause a little finger to develop where a thumb (or its avian counterpart) should be. There are now strong grounds to believe that the antero-posterior morphogen is retinoic acid¹². Implanted beads loaded with this substance mimic the effects of implanted polarizing tissue; it is found in the normal limb bud in an appropriate concentration gradient¹⁴; and, as another paper, published last week in *Nature*¹⁵, shows retinoic acid receptors are present in the limb bud at the critical stages. These receptors belong to the steroid hormone receptor family; like the products of homoeobox-containing genes, they act as DNA-binding transcription factors and are very plausible candidates for a role in the specification of positional values.

It is not obvious exactly how the new findings on *Hox-5* expression in the limb bud fit in with the old story: the *Hox-5* gradient seems to be neither proximo-distal nor antero-posterior, but skewed relative to both axes. Moreover, in spite of all the suggestive observations, we still have no proof that *Hox-5* activation defines the positional values that control the patterning of the limb. That must await studies of transgenics or other mutants, or experiments that combine molecular genetics with microsurgery.

In the meantime, it is wise to be wary of assuming that *Hox-5* activation principally reflects retinoic acid exposure, or, conversely, that it principally reflects time spent in the progress zone. The mechanisms governing the antero-posterior and proximo-distal components of positional value may indeed be more intimately related than our current

theories admit. In regenerating amphibian limbs, for example, exogenous retinoic acid has the extraordinary effect of resetting the proximo-distal positional value of the blastemal cells back to the most proximal state, so that a whole upper arm, forearm and hand may be regenerated from a plane of amputation that cuts through the wrist, giving a monstrously extended limb^{16,17}. Such observations reinforce other hints and rumours that retinoic acid may affect positional values through regulation of HOM gene expression^{12,18,19}. But in any case, it is sure that HOM complexes and retinoic acid and its receptors have a wider and deeper importance in pattern formation than even a certain prophet of universal developmental principles²⁰ had any right to expect. □

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DIAMOND FILMS

Low-pressure nucleation routes

Michael Pinneo

NEW methods for the low-pressure synthesis of diamond in thin-film form are attracting increasing academic and industrial interest. Although diamond is thermodynamically stable only at extremely high pressure, under certain conditions, it can be deposited on a wide variety of materials using chemical vapour deposition (CVD) processes. The mechanisms underlying low-pressure diamond nucleation and growth remain obscure. A new proposal by Bar-Yam and Moustakas, described on page 786 of this issue¹, may help shed light on the critical early stages of diamond film growth, with significant consequences for development of industrial applications using this new material.

Although early reports² of diamond growth within its metastability region were greeted with scepticism, the deposition of

diamond films is now firmly established and can even be performed with an ordinary oxyacetylene welding torch at atmospheric pressure³. Many industrial laboratories, principally in Japan and the United States, are exploring low-pressure diamond growth for a wide variety of applications. A commercial product based on the new material has been released, and others are anticipated soon.

CVD diamond differs from single-crystal natural material mainly in being polycrystalline. Small diamond nuclei initially form on a substrate surface, then grow and coalesce into a fully contiguous film which can be further grown to a desired thickness. Wide variations in film structure and properties can be produced through manipulation of the deposition process. A typical CVD diamond film comprises tightly



Micrograph of CVD diamond film with sharply faceted (111) faces. (Image 16 μ m across.)

packed columnar diamond crystals which have grown up from an underlying silicon substrate (see figure). A growing body of data on CVD diamond indicates that its properties equal those of the natural material in many respects, and surpass it in others. Its extreme hardness, wear resistance, thermal conductivity and electrical resistivity match those of natural diamond^{4,5} and its impurity state can be controlled through the addition of dopants. Diamond diodes and transistors have been fabricated and operate at temperatures in excess of 500 °C.

The low-pressure nucleation of diamond on other materials is poorly understood, but it has been shown experimentally to depend on the substrate's chemistry, surface preparation and surface charge state, among other variables. Nucleation by lattice matching of diamond bonds to an underlying amorphous carbon layer has been proposed⁶, as has nucleation through the creation locally of very high temperatures and pressures by surface bombardment from ionized plasma species. None of these kinetic mechanisms seems adequately descriptive of the observed phenomena. Nor do they give very useful predictions. A more thorough understanding of diamond nucleation is required for the control of interface phenomena such as substrate adhesion, the generation of interface defects and optical scattering properties.

The vacancy-stabilized nucleation mechanism proposed by Bar-Yam and Moustakas¹, on the other hand, could assist in the design of processes to tailor diamond nucleation to specific requirements. For example, their model suggests that the nucleation density of diamond on chemically 'difficult' materials such as tool-steel alloys would be little improved by the etching and polishing methods known to be effective for silicon or molybdenum surfaces. Rather, researchers' attention should be directed to other means that would increase the probability of forming vacancies. This is because, as the authors show, vacancies reduce the energy of diamond film relative to that of graphite, which, of course, would otherwise be the stable form.

The mechanism of metastable diamond growth following nucleation is not understood either, although the experimental

evidence is richer than that for nucleation. Plasma diagnostic methods, including optical emission and absorption spectroscopy, mass spectrometry and Langmuir (electron-density) probes, are disclosing much about the plasma chemistry underlying diamond deposition. Several growth schemes have been proposed, including reactions based on methane⁷ or acetylene⁸ and kinetic selection for diamond through differential etching and bond stabilization by hydrogen at the growth surface. But the experimental evidence does not yet definitively distinguish between the alternative mechanisms, and it is probable that more than one mechanism contributes to CVD diamond growth.

The lack of an accurate description of CVD diamond deposition mechanisms has not dampened enthusiasm for the material's potential applications. Until recently, diamond has only rarely been considered for its usefulness in engineering, owing to its high cost, uncontrolled properties and the inability to achieve large-area coatings using the classical high-pressure synthesis methods. Now that diamond can be created in more useful forms, potential users are re-examining its unique, technologically

important attributes.

But although Bar-Yam and Moustakas predict that defect-free diamonds will be difficult to grow, DeBeers will not breathe easily just yet. Large, low-cost diamonds would be exceedingly useful in industry, not to say aesthetically pleasing, and the incentive to develop suitable growth processes is high. It is interesting to note that growth of thick (0.16-mm) diamond films with defect densities comparable to natural type Ia diamonds has already been reported⁹. □

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GREENHOUSE EFFECT

Gauging water-vapour feedback

Robert D. Cess

IN 1861, Tyndall¹ described the 'greenhouse' effect caused by atmospheric water vapour, pointing out that water vapour transmits a large fraction of the incident solar radiation but strongly absorbs infrared radiation emitted by the Earth's surface. More than a century later Möller² suggested that this greenhouse effect might also act as an amplifying (positive) climate feedback mechanism, a point later emphasized by Manabe and Wetherald³. These and subsequent investigations used theoretical climate models. On page 758 of this issue⁴, however, Raval and Ramanathan cleverly employ satellite measurements to quantify the greenhouse effect and to demonstrate its positive correlation with surface temperature — that is, positive water-vapour feedback.

The explanation for this feedback mechanism is as follows. If the Earth were to warm, for example owing to an enhanced greenhouse effect caused by increasing atmospheric CO₂, the warmer atmosphere should contain more water vapour, which as a greenhouse gas would amplify the initial warming. To put it another way, an anthropogenic increase in one greenhouse gas (CO₂) causes an increase in yet another greenhouse gas (water vapour). It is important to differentiate, in this example, between what is meant by the greenhouse effect, climate forcing and climate feedback.

Raval and Ramanathan's observational elucidation of the greenhouse effect provides a convenient means of making these distinctions within the conventional interpretation of climate change as a two-stage process: forcing and response. The global-mean direct radiative forcing, H , of the surface-atmosphere system is evaluated by holding all other climate parameters fixed. For an increase in atmospheric CO₂, H is the reduction in the top-of-the-atmosphere infrared flux caused solely by the CO₂ increase. The response, within which climate feedback mechanisms are contained, is the ensuing change in climate that is required to restore the top-of-the-atmosphere radiation balance.

Because Raval and Ramanathan's demonstration of water-vapour feedback refers to clear-sky conditions, I will use a hypothetical planet to illustrate the process. This planet has the same global-mean surface temperature (288 K) as Earth, and its atmosphere is cloud free and corresponds to the clear-sky observations employed by Raval and Ramanathan. A further assumption is that absorption of solar radiation by the surface-atmosphere system is invariant to climate change, making

this planet quite different from Earth. Finally, the direct radiative forcing will arbitrarily be chosen to be 4 W m⁻². Although this corresponds to a doubling of CO₂ in the Earth's atmosphere, bear in mind that our hypothetical planet lacks many of the Earth's climate feedback mechanisms; the goal here is to focus solely on clear-sky water-vapour feedback.

By Raval and Ramanathan's definition, the greenhouse effect is $G = E - F$, where E is the infrared surface emission and F is emission at the top of the atmosphere. The base value they determine as 146 W m⁻². Their normalized greenhouse effect is $g = G/\sigma T_s^4$, where T_s is surface temperature and σ is the Stefan-Boltzmann constant. Raval and Ramanathan impressively demonstrate (their Fig. 3a and b on page 760) that g is a function of the surface temperature, this being a consequence of the related dependence of atmospheric water-vapour abundance upon T_s , so that $g(T_s)$ represents the water-vapour feedback mechanism. If g were independent of T_s , there would be no feedback. Furthermore, it is easily shown that

$$F = (1 - g(T_s))\sigma T_s^4$$

where $g(T_s)$ is given in Raval and Ramanathan's Fig. 2.

The hypothetical planet's climate responses are summarized in three stages (see table). The direct infrared forcing (process 1) simultaneously reduces F and increases G , so that the planet emits 4 W m⁻² less energy than it absorbs from the Sun. This imbalance causes global warming that, without water-vapour feedback, increases T_s by 1.09 K (process 2). G is increased by 1.9 W m⁻² because of the enhanced surface emission, and the flux F at the top of the atmosphere is increased by 4 W m⁻², restoring the net balance of incident and outward flux. Further warming then occurs when water-vapour feedback is allowed (process 3).

This simple illustration demonstrates how the direct greenhouse forcing of

Responses of the hypothetical planet's climate system to a 4 W m⁻² direct forcing through, say, increased CO₂

Process	ΔT_s (K)	ΔG (W m ⁻²)	ΔF (W m ⁻²)
1 Direct forcing	0	4.0	-4.0
2 Response with fixed $g=0.327$	1.09	1.9	4.0
3 Further response to variable g	0.64	3.6	0
Total Response	1.73	9.5	0

4 W m⁻² is first amplified to 5.9 W m⁻² through temperature feedback (process 2) and then to 9.5 W m⁻² by water-vapour feedback (process 3). The combined forcing and feedbacks thus increase the clear-sky greenhouse effect from its initial value of 146 W m⁻² to nearly 156 W m⁻². Note that water-vapour feedback amplifies surface warming by roughly 60 per cent.

On a further point, Raval and Raman-

athan show that their observational interpretation of water-vapour feedback is compatible with results from climate models, and it is worth expanding on this point. Our recent intercomparison⁵ of 14 atmospheric general circulation models (GCMs) shows significant disagreements amongst the models' global-mean dF/dT_s values. This, however, is primarily caused by differences in the depiction of cloud feedback. When output from the GCMs is processed in the same way as the data used by Raval and Ramanathan, to average clear-sky regions separately, there is quite good agreement in their clear-sky dF/dT_s values: the average for the 14 GCMs is $2.38 \pm 0.16 \text{ W m}^{-2} \text{ K}^{-1}$, which compares favourably with Raval and Ramanathan's observational value, $2.31 \text{ W m}^{-2} \text{ K}^{-1}$, derived using the equation above. But if the water-vapour feedback is suppressed by making $g(T_s)$ fixed in the equation, the observational value would

be $3.67 \text{ W m}^{-2} \text{ K}^{-1}$, significantly exceeding the GCM values.

As Raval and Ramanathan emphasize, their observed water-vapour feedback has a very simple explanation: it is dominated by the saturation vapour pressure of water through the Clausius–Clapeyron equation. The same explanation would seem to apply to the GCMs. With all the uncertainties in our knowledge of climate processes, especially cloud feedback⁵, it is gratifying that at least one such process has a simple explanation. □

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DNA STRUCTURE

The turn of the quadruplex?

Maxim Frank-Kamenetskii

WHILE molecular biologists are still wondering how to deal with triplexes, even more unusual DNA structures are surfacing. On page 825 of this issue, Sundquist and Klug¹, demonstrate that artificial DNA molecules, which have one blunt end and an overhanging single-stranded TTGGGGTTGGGG tail at the other end, form remarkably stable dimers in solution. Their observation may have biological implications because these tails are characteristic of the ends of *Tetrahymena* chromosomes, and the ends of chromosomes (telomeres) in other species, including *Homo sapiens*, carry very similar sequences. Chemical probing of the dimers ruled out the possibility of a duplex forming between the tails, and led Sundquist and Klug to the conclusion that the tails are attached to each other by the following unusual mechanism. First the tails fold back, forming hairpins stabilized by Hoogsteen G:G pairs². Then two such hairpins associate forming a stack of four G4 tetrads (See Fig. 4b of their paper, page 829). The thymines serve as linking elements in the construction.

For decades, G-rich oligo- and polynucleotides have been notorious because of their ability for inter- and intramolecular aggregation. Gellert *et al.*³ were the first to suggest that GMP aggregates in the form of G4 tetrads, in which four guanines cohere through Hoogsteen pairing. But comprehensive studies of the G-aggregates were hampered by the lack of adequate experimental tools. Sen and Gilbert⁴ applied the chemical-probing technique to this problem, and demonstrated that G-tetrads were formed in

aggregates of G-rich strands.

Chemical probing is becoming increasingly popular in studies of unusual DNA structures, such as cruciform, the Z form and the H form. In this method, a DNA carrying an unusual structure is subjected to a chemical reaction with known specificity. The DNA is end-labelled and the reaction sites are converted into chain breaks by treatment with piperidine, just as in the standard Maxam–Gilbert protocol for DNA sequencing, with subsequent separation of the DNA fragments in a denaturing gel. In so doing one localizes the sites of DNA modification by the reagent at sequence resolution. Sen and Gilbert followed the methylation of the guanine N7 position and found that the guanines in G-tracts were not methylated in the aggregates. This provided strong evidence that a structure had been formed in which the guanines were involved in Hoogsteen pairing. The aggregates studied by Sen and Gilbert were tetramers, and in G-tetrads all the N7 positions participate in hydrogen bonding, hence the conclusion that the G-tetrads were responsible for aggregation.

The coherence of telomeres carrying single-stranded tails was first described for *Oxytricha*^{5,6}. In these studies DNA molecules were double-tailed, however, so it was difficult to arrive at any definite conclusion about the nature of the aggregates. Sundquist and Klug prepared single-tailed DNA molecules (with *Tetrahymena* telomeric tails TTGGGG–TTGGGG), which enabled them to show beyond any doubt that dimers were formed, not the tetramers postulated by

Sen and Gilbert.

At the same time methylation experiments clearly indicated that the N7 positions of all tail guanines from both monomers are completely protected in the dimer, exactly as predicted by Sen and Gilbert. Sundquist and Klug explain their data in terms of the structure shown in Fig. 4b of the paper. In contrast with the model of Sen and Gilbert, all adjacent chains in the quadruplex are antiparallel. This implies the formation of not only inter- but also intramolecular quadruplexes. Panyutin *et al.*⁷ have recently observed periodic methylation patterns for 27- and 37-long oligo(G), which they interpreted as an indication of the folding of molecules into an intrastrand quadruplex with three loops forming bends. These loops corresponded to the three maxima on their methylation patterns.

The structure proposed by Sundquist and Klug provides a model of recognition and association of two identical, rather than complementary, DNA sequences. It is tempting to speculate that the association of telomeric tails is the first step towards the recognition of homologous DNA molecules by each other in meiosis. This speculation, however, is questionable because we now know the main biological function of telomeric tails: they serve as primers for telomerase, which elongates the ends of chromosomes.

So why do people search for additional functions? The homologous recognition of DNA molecules may be mediated by proteins. This is most probably true for present-day forms of life. But the fact that telomerase carries an RNA molecule, part of which is complementary to the telomeric tail, has led to the assumption that telomeres are molecular fossils of the RNA world⁸. There were no proteins at the time and the ancient RNA had only itself to rely on. More data are accumulating to suggest that the most fundamental processes in eukaryotes conserve ancient principles inherited from the RNA world. Who can guarantee that the principle of homology recognition proposed by Sundquist and Klug is not of this category? □

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■ Editorial note: single-stranded telomeric sequences have been shown to fold back on themselves to form cyclic guanine tetrads (J.R. Williamson, M.K. Raghuraman & T.R. Cech *Cell*; in the press).

Four legs to stand on...

Henry Gee

IN *An Introduction to Dogs*, Ogden Nash wrote that man's best friend is distinguished by having "a tail on one end", adding that "up in front he has teeth" and, moreover, "four legs underneath". A more succinct definition of a tetrapod is hard to imagine, but its simplicity disguises the many assumptions, often unwarranted, that are used to reconstruct the history of life. The explosion of popular myths was a recurrent theme of a recent meeting*.

One assumption is that the first tetrapods had five-toed limbs. For example, the early tetrapod *Ichthyostega* from the Upper Devonian of Greenland is often reconstructed with five digits per limb, even though the bones of its feet have not been found. But the early amphibian *Tulerpeton* from the Soviet Union is now known to have had six digits per limb, and there are indications from material collected in 1987 and now under study in Cambridge that *Acanthostega* from the Upper Devonian of East Greenland (J. A. Clack and M. I. Coates, University of Cambridge) may also have had more than five. It now seems that the earliest tetrapods had a variable number of digits: pentadactyly is not a primitive feature of tetrapods, but a derived character that became established later on, after a period of evolutionary experimentation. The implication is that it cannot be used as a primitive feature against which to gauge the subsequent phylogenetic pattern of the tetrapods.

Aquatic origins

Other long-held assumptions about vertebrate life now seem to have been based more on phylogenetic inference than on actual fact. The presence of lungs in lungfish before the Permian, for example, is an assumption unsupported by anything besides the principle of parsimony (K. S. Thomson, Academy of Natural Sciences, Philadelphia). As with the primitively pentadactyl limb, it cannot be said with certainty that lungs were primitively present in lungfish, despite their name. Similarly, the common supposition of an aquatic habit in a stocky mammal-like reptile called *Lystrosaurus* is based on wishful thinking (G. M. King, South African Museum, Cape Town). That the many reconstructions of this well-known and common fossil form portray it as anything from hippo-like to a fully aquatic animal, complete with flippers, illustrates the power of popular reconstructions to pass as conventional wisdom.

Whereas it is hard to find any feature of

Lystrosaurus that unequivocally points to an aquatic lifestyle, ichthyosaurs were so completely adapted to the marine habit that judging their relationships with other tetrapods is extremely difficult. This task is made even harder by another kind of wishful thinking — the existence of fine impressions of fins on a number of ichthyosaur fossils turns out to owe more to the preparators' imaginations than fact (D. Martill, Open University, UK). Although many ichthyosaur fin impressions are undoubtedly real, those of dorsal fins are just the kind of appealing features that are carried over into reconstructions.

Vertebrate life began in the sea, but

beyond that the topic of vertebrate origins remains contentious. The problem is that any proposal to explain vertebrate origins is, inevitably, rooted in arguments about homology and structure that some will always dismiss as wishful thinking. It now seems that Garstang's ideas about vertebrate origins command respect chiefly through their perpetuation in numerous textbooks.

This explains why an obscure group of echinoderm-like fossils known as calcichordates was until recently regarded as marginal. But the presentation of no less than three papers describing new calcichordate forms (R. P. S. Jefferies, A. P. Cripps and P. Daley, Natural History Museum, London) signals a revival of interest in the general question of vertebrate origins (H. Gee, *Nature* 340, 596–597; 1989).

The status of conodonts in the debate

...for Devonian vertebrates

Per Erik Ahlberg

THE Middle Palaeozoic, comprising the Silurian and Devonian periods, was an important phase in the evolution of early vertebrates; they increased rapidly in numbers and diversity, jawed fishes began to replace the jawless forms and the first land vertebrates (tetrapods) appeared. Some of the reports at a meeting in Estonia* provided fresh evidence for re-interpretation of the environment inhabited by the earliest tetrapods and their fish ancestors.

Estonia was an appropriate venue for the conference. Fossil-rich Silurian and Devonian rocks underlie large parts of the Baltic States, and the Devonian vertebrate faunas of the region are among the finest and most diverse in the world. Because of the political thaw, some of the fossil sites are now accessible to Westerners for the first time in 50 years. On several occasions it was clear that Soviet and Western researchers had already more or less independently reached the same conclusions — none more so than with the environmental re-interpretation of the Old Red Sandstone.

Most Devonian fossil fish faunas, as well as the earliest tetrapods, occur in a widespread group of sedimentary facies collectively known as the Old Red Sandstone, or ORS. Some of these sediments are cross-bedded channel sands, others are finely laminated still-water deposits. They often contain plant fragments, lack typical marine invertebrates and have traditionally been interpreted as being freshwater in origin. Recently, many of the ORS fishes have turned up elsewhere in fully marine sediments, which has led to the sugges-

tion that the ORS itself might be partly marine.

There is far from general agreement over the 'marine hypothesis', but two of the papers given at the meeting lend it considerable support. Daniel Vézina (Parc de Miguasha, Canada) pointed out that the concentrations of calcium, magnesium and iron in the Canadian ORS of Escuminac (Scaumenac) Bay, an important Devonian fish locality, are typical of marine deposits. Visvaldis Kuršs (Latvian University, Riga) revealed that the extensive Baltic Middle and Upper ORS contains lingulid brachiopods and abundant phosphate deposits, both signs of marine influence. The Baltic ORS is most probably deltaic in origin; the precise environmental context of Escuminac Bay is less certain.

The re-interpretation of the Old Red Sandstone has important implications for our ideas about the origin of tetrapods, given the general assumption in the past that this event occurred in a freshwater environment. Both Escuminac Bay and the Baltic ORS contain fossils of panderichthyids, enigmatic lobe-finned fishes that are probably very close to the origin of tetrapods. Oleg Lebedev (Palaeontological Institute, Moscow) told the meeting that the Russian Upper Devonian tetrapod *Tulerpeton*, one of the earliest known, comes from deposits which are very probably deltaic or lagoonal. Could it be, then, that the first tetrapods emerged from the mudflats and brackish waters of Devonian deltas? □

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* 37th Symposium on Vertebrate Palaeontology and Comparative Anatomy, Leicester, UK, 18–22 September, 1989.

* The Second International Colloquium on the Middle Palaeozoic Fishes, Tallinn, Estonia, 11–15 September 1989.

SCANNING ATOMIC MICROSCOPY

over vertebrate origins is similarly open to dispute. Until recently, teeth were all that betrayed the existence of these animals. Although common enough to be used as biostratigraphic marker fossils, the absence of any other tissue made any systematic treatment impossible.

The discovery in Scotland of the fossil of a long, worm-like animal with recognizably conodont teeth at one end has sparked yet more dispute: they could be chordates related to the hagfish *Myxine* (R. J. Aldridge, University of Leicester, and D. Briggs, University of Bristol), and there is even some evidence for the presence of chordate-like segmental muscle blocks. But other palaeontologists interpret these structures differently, and argue that the conodonts have affinities with aplousophoran molluscs or nemertean worms, or that they are so different from other forms that they constitute a distinct phylum.

Genetic patterns

Genetics might provide one way to dispel palaeontological myths once and for all. The realization that distinctive homoeobox genes in animals from different phyla share extensive sequential and structural homology (N. H. Patel *et al. Cell* **58**, 955–968; 1989) was one of the main talking points at the meeting. Palaeontologists have often been wary of claims that phylogenetic problems can better be unravelled by analysis of molecular sequence data than through the fossil record. These data come from structural genes and proteins, and their interpretation is plagued by the same problems that beset anatomical evidence. A protein, after all, is just as much a part of an animal's anatomy as is its skeleton.

But homoeobox genes are concerned with the elaboration of anatomical structures rather than simply the differences between them. The unique body plans that are used to characterize phyla are thought to be rooted in different ontogenetic programmes, but how these differences are established at the molecular level is unclear. One idea is that patterns of duplication in homoeobox genes among various deuterostome taxa (P. Holland, University of Oxford) may add useful evidence with which to reconstruct vertebrate phylogeny, and to judge between competing hypotheses based on purely anatomical evidence.

Ogden Nash's dog had a tail on one end, in common with ichthyosaurs, tunicate tadpole larvae and (arguably) calcichordates and conodonts as well. And *Acanthostega* and *Tulerpeton* had precisely four legs underneath, never mind how many digits they sported on the ends. Homoeobox genes may tell us why. □

Henry Gee is on the editorial staff of *Nature*.

Variations on an original theme

C. F. Quate

In the nine years since its invention, the scanning atomic probe microscope has been adapted in numerous ways so that it can be used to trace not merely surface topography but also surface properties with remarkable resolution. The adaptation, described by Weaver *et al.* on page 783 of this issue¹, exploiting the thermoelectric effect to identify individual species, exemplifies the ingenuity now applied in the field.

Early in the 1980s Heini Rohrer and Gerd Binnig discovered that a single atom at the end of a sharp tungsten needle can probe the electronic structure of smooth surfaces. When placed close to the surface and scanned mechanically over it, their atomic probe gave sufficient resolution to define the spatial positions of individual surficial atoms with great precision. But surface scientists need to know more than the location and the chemical nature of the atoms; they also want to know about the environment surrounding the site, the size of the atom's magnetic dipole moment, the size of the electric dipole and much more.

In their new report, Weaver *et al.*¹ outline a strategy for acquiring information of this kind, exploiting the thermal voltage generated when a substrate is heated with the energy absorbed from a laser beam. It is well known from optical spectroscopy that such heating is selective: different species absorb energy at dif-

ferent wavelengths, so that the result of scanning a heterogeneous surface with a particular wavelength is a variation in the temperature and the chemical potential². As Weaver *et al.* point out, the tip of a scanning probe and the atoms of the sample are in contact owing to the overlap of their electronic wavefunctions. Electrons flow through the (resistive) contact to equalize the differing chemical potentials and so generate a voltage that is characteristic of the surface atomic species (see figure, over).

The strong interaction between electron waves and optical waves was previously exploited by Gimzewski *et al.*³, who measured the optical emission from the tunnelling region. Light emission, stimulated by the tunnelling electrons, was first reported by Lambe and McCarthy⁴. It is the result of an inelastic tunnelling process, directly related to the inverse photoelectric effect⁵ in which the electrons in the gap deliver energy to the optical modes. Gimzewski *et al.* examined a silver film deposited on a silicon substrate and found that the light was associated with surface plasmons (charge density oscillations). The scanning tip perturbs these modes in such a way that they radiate from the vicinity of the tip and the optical emission yields a spatial map useful for characterizing nanometre-sized features.

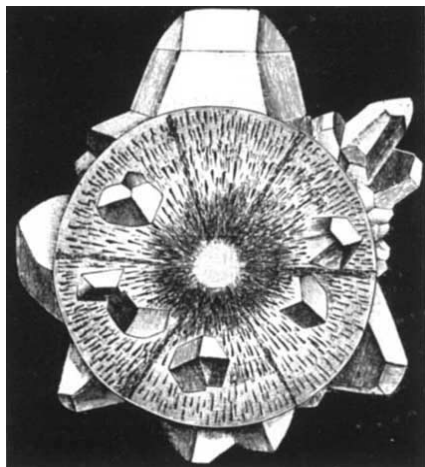
How can the techniques be extended to

100 years ago

REMARKABLE HAILSTONES

ON p. 43 of the present volume of *Nature* the following extract is given from a paper by Prof. Houston in the *Journal of the Franklin Institute*: — "On some of the hailstones, though not on the majority of them, well-marked crystals of clear transparent ice projected from their outer surfaces for distances ranging from $\frac{1}{8}$ to $\frac{1}{4}$ of an inch. These crystals, as well as I could observe from the evanescent nature of the material, were hexagonal prisms with clearly cut terminal facets. They resembled the projecting crystals that form so common a lining in geodic masses, in which they have formed by gradual crystallization from the mother-liquor. They differed, however, of course, in being on the outer surface of the spherules."

It is evident from Prof. Houston's paper that this form of hail was unknown to him as it must also have been unknown to many who have propounded theories as to the formation of hail which will not account for it, a service may be rendered to meteorology by the reproduction of the exquisite lithographs of this form of hail given in Prof. Abich's paper, "Ueber krystallinischen Hagel im Thiralethischen gebirge," published at Tiflis in 1871. The hailstones represented all fell on June 9 (21), 1867, at Bjeloi Kliutsch, a village about twenty

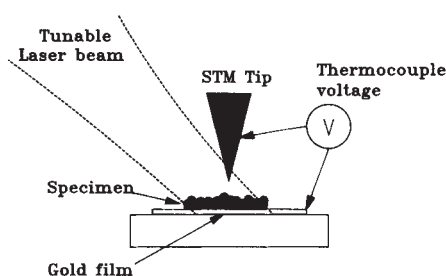


miles south-east of Tiflis, and 12,425 feet above sea-level.

Theories of the formation of hail are almost innumerable. Some — like Prof. Schwedoff's, that hailstones come from interplanetary space — are very droll; but the subject is a very difficult one, and one upon which I do not know of a single good treatise in our language. Possibly, the reproduction of these figures may induce someone to prepare an exhaustive memoir.

G. J. SYMONS

From *Nature* **XLI**, 134–135 (1889).



Schematic of the scanning atomic probe used by Weaver *et al.*, exploiting laser-induced thermoelectric voltages. (Courtesy of H. K. Wickramasinghe.)

measuring surface magnetization? Aware of the lack of coupling between the magnetic system and the tunnelling electrons, Allenspach and Bischof⁶ measured the spin polarization of the secondary electrons that are emitted when primary electrons from the tip impinge on the surface of the sample. The spin polarization is a direct measure of the surface magnetization and the scanning probe used in this way produces a map of the magnetic surface. Unfortunately, it was necessary to retract the tip from the surface and operate in the field-emission mode⁷ (not a tunnelling process) so that the secondary electrons could escape from the surface and reach the detector without colliding with the tip. This procedure results in poorer resolution and precludes measurement at single atomic sites.

To achieve atomic resolution, the tip must be moved close to the sample and into the regime where the electrons tunnel from the sample to the tip. But in that regime the tunnelling current is not responsive to the surface magnetization. Or is it? In some special cases there is evidence of coupling between the tunnelling current and the paramagnetic spin of the electrons. Manassen⁸ has used this spin-dependent tunnelling to image paramagnetic spin centres in silicon oxide films. The electron spin resonance was tuned to 500 MHz and the component of tunnelling current at this frequency was measured while the probe was scanned through the spin centre. The coupling mechanism is not completely understood and further work will be needed to clarify the physics before the technique can be extended to other systems. □

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MID-OCEAN RIDGES

Propagating rifts exposed

Ken C. Macdonald

MID-OCEAN ridges, where plates of oceanic lithosphere separate, are not invariant features. Major changes in spreading direction require the ridges to reorganize themselves. Spreading centres can 'jump' from one location to another. How do these changes occur? And what causes the large-scale sea-floor patterns that are oblique to both spreading centres and fracture zones? In a *tour de force* of high-resolution observation of the sea floor^{1–3}, M.C. Kleinrock, R.N. Hey and R.C. Searle present the most extensive, detailed study yet of a propagating rift

more evidence of recent volcanic eruptions than the new propagating rift^{1–3,6} — they seem to go out "with a bang, not a whimper". Extensive fields of fresh, glassy lava suggest that the volcanic/magmatic budget of the failing rift is sustained some time after spreading has begun to slow down. Thus the availability of magma relative to the rate of spreading and extension may temporarily be enhanced.

Conversely, there is a dearth of volcanism at the new propagating rift tip^{1–3,5,6}. The tip of furthest advance is the

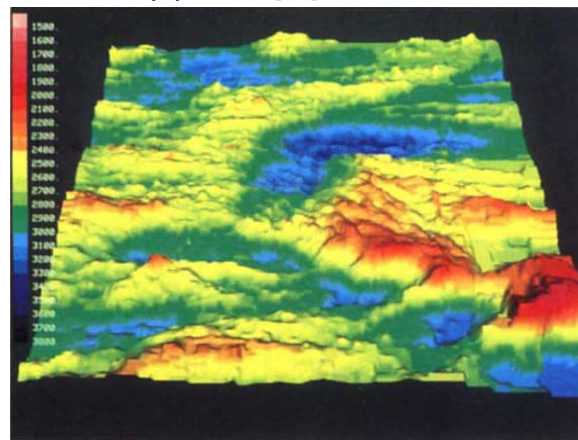


FIG. 1 Shaded relief image of the propagating rift at 95° W on the Galapagos Spreading Centre studied by Kleinrock and colleagues^{1–3} (produced by S.P. Miller at the University of California, Santa Barbara Seafloor Image Processing facility using SeaBeam data from ref. 6). The rift valley in dark blue towards the top of the image (North) is the propagating rift. The series of four basins (blue) in the near field are the failing rift (left) and several failed rifts (right). The propagating rift is lengthening to the left (West) at a rate of 52 mm yr⁻¹.

system (Fig. 1), providing the answers to some of these questions.

For over ten years, Hey and others^{4,5} have said that rift propagation through the lithosphere can explain all these phenomena. In this conceptually simple model, a new mid-ocean ridge develops perpendicular to a new spreading direction and propagates by lengthening into existing rigid oceanic lithosphere. Meanwhile, the old or 'doomed' spreading centre ceases spreading as it is overtaken by the new one (Figs 1 and 2). A V-shaped wake results, with the boundary between old and new lithosphere forming one limb of the V, and the boundary between new lithosphere and the failed spreading axis forming the other limb. The shape of the V can be used to determine the propagation speed — often about the same as the half-spreading rate of 10–100 mm per year.

The propagating rift model has survived the closest scrutiny possible with available high-resolution tools. But some of the new observations^{1–3} are surprising. For example, the failing rifts show much

tectonic tip where crack propagation into the lithosphere occurs along pre-existing normal faults once active at the failing spreading centres one million years ago. The first signs of recent volcanism occur at the initial volcanic tip, 6.5 km behind the tectonic tip. This is followed by the tip of the axial neovolcanic zone 4.5 km behind, and the full-rate tip another 10 km behind¹. Developing, transient magma chambers may feed the discrete volcanoes of the neovolcanic zone⁷, whereas the initial volcanic tip may be fed by dykes which sporadically shoot many kilometres ahead (west) of the neovolcanic tip, or directly up from the mantle. The 21 km between the tectonic and full-rate tips, and the average propagation rate of 52 km per million years, mean that about 400,000 years pass before the propagator evolves into a mature spreading centre.

In the migrating transform zone that links the propagating and failing spreading centres (Fig. 2), there is no indication of a true transform, which would have north-south trending structures (Fig. 1). Instead there is a 20-km wide zone in

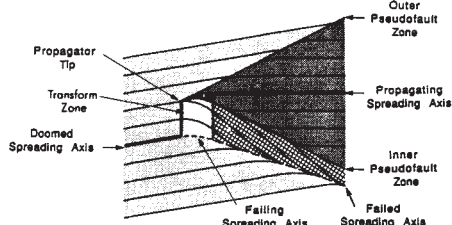


FIG. 2 Idealized model of rift propagation after Hey and colleagues⁶. Dark stipple represents crust created at the propagating rift; light stipple, old crust produced at the doomed spreading centre; cross-hatching, transform zone crust transferred from the plate on the north to the plate on the south.

which crust has been rotated clockwise 40° – 60° , perhaps by a 'bookshelf' mechanism in which blocks of finite width rotate¹⁻³. As a result, the transform zone and its fossil remnants along the inner pseudofault are so pervasively chopped, extended, sheared, compressed and rotated, that not a single outcrop of rock could be found in place during a series of submersible investigations in the migrating transform area. Even the crests of ridges were buried in fresh talus (rock fragments associated with active faulting), as if the talus had rained down from above. Thus the propagating rift and migrating transform zone are magmatically starved and severely tectonized, whereas the failing rifts are magmatically robust with only modest tectonic deformation¹⁻³.

These observations also have important implications for overlapping spreading centres (OSCs) which are ubiquitous

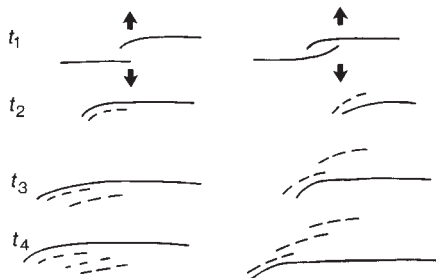


FIG. 3 How the evolution of propagating rifts (left) and overlapping spreading centres (right) differ (after refs 1 and 10). At time t_1 , both spreading centres at the propagating rift and OSC are shown, thereafter only the propagating or lengthening spreading centre is shown (solid line). At a propagating rift, where there is little or no overlap between the two spreading centres, the spreading centre repeatedly cuts outside the pre-existing ridge tip (broken line). At an OSC or at a propagating rift that is also an OSC, the spreading centre cuts inside the pre-existing ridge. The tectonic evolution can become complex when the propagation direction changes sporadically and when the ridge axis discontinuity is an OSC only part of the time. Bold arrows, direction of plate motion.

along most mid-ocean ridges that spread faster than 60 mm a year (full rate)⁸. At OSCs, the two ridges overlap *en echelon* by approximately three times the offset distance (Fig. 3). Most are also small propagating rifts, although they do not propagate into rigid lithosphere, and thus do not produce major shifts and jumps in the spreading axis. They merely rearrange the locus of spreading within the plate boundary zone⁹. Also, some OSCs are relatively stationary and thus are not propagating rifts. Conversely, many propagating rifts are OSCs in which the failing and propagating rifts overlap much more than they do at the Galapagos 95° W propagator; for example, the East Pacific Rise at $20^\circ 40'$ S (ref. 10). Whether a propagating rift has little overlap as at 95° W, or is an OSC with large overlap may control the way it evolves, as shown

in Fig. 3. At the 95° W propagator the neovolcanic tip is clipped off as the propagator surges past it to the north or "to the outside" (refs 5,6) and is then rotated in the migrating transform zone¹. In contrast, an OSC evolves in such a way that the new ridge tip usually cuts to the inside of the old ridge tip, or to the south in this example⁹.

The tectonics can get even more complicated if the propagating rift changes its direction of propagation episodically and is also an OSC during some of its history; such is the case¹⁰ on the East Pacific Rise near $20^\circ 40'$ S. Yet the complexities can be understood in terms of simple variations of the basic model for rift propagation. The importance of these migratory discontinuities along the mid-ocean ridge is considerable, not only because they provide answers to the fundamental questions posed at the beginning of this article, but

also because their off-axis V-shaped wakes disrupt the structure and geochemical composition of as much as 20 per cent of the ocean floor⁸. □

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SOCIAL INSECTS

Who are the drone police?

Jon Seger

SOCIAL-insect workers may be the ultimate reproductive altruists, but their altruism can be less than pure. In some species of social Hymenoptera, for example, workers substitute their own haploid (male-producing) eggs for those of the queen, thereby achieving direct personal fitness in addition to the indirect inclusive fitness they achieve by helping to rear reproductive sisters. Worker reproduction is taxonomically widespread, but far from universal. On page 796 of this issue¹, Ratnieks and Visscher show that worker honeybees efficiently detect and destroy eggs laid by other workers. Thus worker-laid males may be rare in *Apis*

mellifera not because the queen objects to them, but because the workers cannot agree who should do the laying.

Worker reproduction has often been used to test evolutionary arguments and models. Nearly 100 years ago, Weismann² and Spencer³ cited laying workers as evidence supporting their positions on the relative importance of darwinian and lamarckian evolution. Laying workers also figured prominently in Hamilton's^{4,5} development of inclusive-fitness theory and in Trivers and Hare's analysis⁶ of the sex ratios of social insects. During this century our image of the social insect colony has evolved from that of a quaint



Laying in vain — the eggs of worker honeybees are likely to be destroyed by other workers.

Photo taken by P. Kirk Visscher

class-structured society (as the established terminology will forever remind us), to that of a remorselessly efficient super-organism, to that of an endlessly squabbling nuclear family, and the conceptual emphasis has shifted from issues of reproductive altruism to issues of reproductive conflict.

Workers in most species of social Hymenoptera have potentially functional ovaries, but the average degree of ovarian development varies widely among and within species⁷. Worker laying has become an act of care-giving in some species of ants and bees, where specialized trophic eggs are fed directly to developing larvae. But in many species, including some with trophic eggs, fully viable worker-laid eggs can also be produced. According to theory, workers should value sons (to whom they are related by $r = 1/2$) over brothers ($r = 1/4$); and, in species with single queens who mate only once, they should even prefer rearing nephews ($r = 3/8$) to rearing brothers. Consistent with theory, worker reproduction is believed to account for substantial proportions of males in some species⁸. But this merely sharpens the question — why not in all?

One answer is that the queen 'suppresses' worker reproduction. Colonies in many species produce flushes of worker-derived males following the death of the queen and, at a mechanistic level, the presence of a queen can inhibit the development of workers' ovaries⁸. But, at an evolutionary level, does this kind of 'control' equate to coercion or merely to signalling? In very small colonies, queens can bully workers and eat their eggs, but in the large colonies of socially advanced species such direct control is probably impossible. Theorists continue to search for devious (but evolutionarily stable) ways in which queens might coerce workers they seldom meet, but so far no really convincing mechanisms have been proposed.

Honeybees do not produce trophic eggs, yet a substantial minority of workers show slight ovarian development, with up to a few per cent showing development judged sufficient for egg-laying⁸. However, only a tiny minority (about 0.1 per cent) of males were worker-derived in a recent study, by Visscher⁹, of 11 colonies in which all workers were heterozygous for a visible genetic marker that revealed half of their offspring. This implies that few of the (presumptive) worker-laid eggs survived to adulthood. What happened to them?

Enter the self-appointed 'worker police'. If the queen mates only once then any worker prefers her sister's sons ($r = 3/8$) to her mother's sons ($r = 1/4$), and she therefore ought to be willing to rear worker-laid males in general, not just her own sons. But if her mother mated with

several males, most of the worker's nest-mates are half-sisters ($r = 1/4$) who produce half-nephews ($r = 1/8$); only a few are full sisters ($r = 3/4$) who produce full nephews ($r = 3/8$). Thus a worker's average relatedness to worker-derived males can drop well below her relatedness to queen-derived males; given a cost-effective means of doing so, she ought to block the production of worker-derived males^{10,11}. Unless they could distinguish eggs or larvae that would give rise to full nephews from those that would give rise to half-nephews, all workers should agree that all worker-laid eggs (except their own, of course) must go. It is ironic that the queen's interests are also served by the universal back-stabbing that ensues.

Honeybees meet the key assumption of this model, because queens typically mate 10–20 times¹². More generally, the model seems to be consistent with a pattern in which species with queens known to mate only once have substantial levels of worker reproduction (bumblebees and stingless bees for example), whereas species with queens known to mate several times have little worker reproduction (honeybees, yellowjackets)¹¹. The report by Ratnieks and Visscher shows that the destruction of worker-laid eggs is a plausible mechanism of worker policing in honeybees. Workers removed almost all worker-laid eggs that were presented to them, but only a modest proportion of queen-laid eggs, and this discrimination was apparently not based on relatedness.

Like the thought police in George Orwell's *Nineteen Eighty-Four*, the drone police may be watching you at any time, because anyone might be a member. But perhaps it is not quite so simple. The experiments of Ratnieks and Visscher raise questions about both the origins and the consequences of differences between workers, most obviously between workers with and without developed ovaries, and in principle also between other groups that have yet to be defined. □

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A clean press

THE traditional method of cleaning things is to agitate them in a liquid, detaching the dirt particles by viscous drag. It is extremely inefficient. The liquid boundary layer at a solid surface is always static, so that even the most violent agitation scarcely reaches the intimate surface where the dirt particles lurk.

The basic problem as Daedalus sees it is that liquids are incompressible. The ideal mode of agitation would expand the fluid away from the surface to be cleaned, dragging the dirt with it; but this is the one flow pattern forbidden to a liquid. So Daedalus proposes to use a compressible cleaning solvent — a supercritical fluid. Many supercritical fluids, like carbon dioxide, are already used industrially in various extractive processes. They are surprisingly good solvents. As a dry-cleaning fluid, carbon dioxide should dissolve quite a lot of dirt directly and suspend the rest.

A supercritical washing machine will need sturdy construction to withstand some 70 atmospheres of internal pressure. But by repeatedly raising and lowering that pressure in cloud-chamber fashion, it will force its supercritical solvent into a gentle but ideally efficient 'breathing' mode of agitation. Except for one mathematically inevitable fixed point, every element of the fluid will be driven in an expansive, radial flow pattern ideal for detaching dirt from solid surfaces.

Supercritical dry cleaning will be ecologically impeccable. No detergent will be needed or water wasted, carbon dioxide is more benign than any chlorocarbon and will clean amazingly quickly. Drying will be no problem, either. The carbon dioxide expands back to dry gas when the pressure is finally released. Simultaneously, all the suspended and dissolved dirt is precipitated. It will be filtered from the gas stream for possible re-use; maybe as fertilizer, or in the cases of foodwaste from dish washing, animal feed. Best of all, the cleaning effect can be intensified at will simply by increasing the depth of the pressure cycle.

For the ultimate in power laundering, the pressure could simply be released in one big blast, blowing the dirt away. Such forceful cleaning could open up whole new fields of renovation like instant paint stripping and derusting. A suitable supercritical solvent mixture might even strip printing ink from paper.

At last the major waste product of our bureaucratic, computer-ridden civilization could be effortlessly recycled. Printouts, memos, pamphlets, junk mail, newspapers, all could be instantly erased for re-use. Folds or creases could well be ironed out in the violence of the expansion, leaving pristine new paper. The separated dirt could be neatly recycled into more printing ink.

David Jones

Sampling the lithosphere

SIR—Two recent contributions to *Nature*^{1,2} highlight the importance of the continental lithospheric mantle to contemporary geochemistry. It is of interest both because it may represent a significant repository of incompatible trace elements that must be included in any model of the Earth's evolution, and because it contains old segments of the upper mantle, often with distinctive isotope ratios that are important tracers in studies of mantle geodynamics. As with all geochemical reservoirs, however, there is the fundamental problem of obtaining a representative sample, and this is particularly acute for the continental lithosphere.

Mantle xenoliths transported to the surface in alkali basalts and kimberlites remain the least ambiguous. They have yielded much information on the mineralogy and composition of the uppermost mantle, but they are strikingly diverse. In particular, many of the garnet-bearing peridotites are unusually depleted in major elements, including iron, with the result that they are on average 1 per cent less dense than most mantle samples³. They also seem to be concentrated beneath the older cratonic areas, and to have the more extreme isotope ratios. By contrast, spinel-bearing peridotites occur in geologically young terrains, and both their isotope ratios and their major-element compositions are probably similar to the uppermost mantle in oceanic areas. One inference is that lithosphere generating processes are no longer the same as they were in the Archaean, but whatever the causes, the diversity of mantle xenolith suites demands that a range of rock types be included in any estimate of lithosphere composition.

But are there other ways of sampling the lithosphere? Our understanding of the geochemical variations in the asthenosphere is dominated by the analysis of oceanic basalts which provide an average sample of the trace element and isotope characteristics of their source regions. Arguably a similar approach can be applied to the lithospheric sources of continental magmatism and although debates concerning the effects of crustal contamination continue, there is increasing confidence that many continental basalts are derived from mantle source regions that are both distinct from the asthenosphere and of similar age to the overlying crust. These observations are consistent with an origin within the continental lithospheric mantle, whereas variations in trace-element abundances imply that such sources can be stabilized by a variety of processes. These processes are as likely to occur at convergent plate margins⁴ as in extensional environments, contrary to the implication of Jochum *et al.*

*al.*¹ (more firmly stated by Sun and McDonough⁵) that most of the lithospheric mantle is generated at divergent plate margin or intraplate tectonic regimes.

It is becoming increasingly clear that progress towards a fuller understanding of the subcontinental lithospheric mantle requires integration of the information derived from both xenoliths and basaltic magmas. The new analyses of spinel peridotites are most welcome and an important contribution to this goal. Considering their particularly fertile major-element compositions and their trace-element similarity to oceanic basalts, they are, however, unlikely to be representative of the most distinctive features of the continental lithospheric mantle.

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Jochum *et al.* reply—Rogers *et al.* suggest that the continental lithospheric mantle (CLM) may grow at convergent plate margins, although evidence for this process has not been found in spinel lherzolite xenoliths¹. Their comments lead to two important questions in first, how representative are spinel lherzolite xenoliths of the CLM, particularly in older cratonic regions; and second, can continental basalts be used to characterize the composition of the CLM? We chose spinel peridotite xenoliths for this study because they are the freshest samples of continental lithospheric mantle available and therefore provide direct evidence on the lithospheric mantle's incompatible-trace-element composition. These xenoliths, unlike most garnet peridotites that are carried by kimberlitic magmas, have not been significantly affected by host contamination. The xenoliths we described cover a range of major and trace-element compositions. However, as Rogers *et al.* point out, they may not be representative of the continental lithospheric mantle in cratonic regions. Nevertheless, our conclusions¹ concerning the growth and evolution of the CLM also hold when data for garnet peridotite xenoliths are considered⁶. Furthermore, comparisons of spinel and garnet peridotite suites show that they follow similar chemical trends.

Our arguments are not based on absolute abundances of major and/or incompatible trace elements, but on ratios of incompatible elements with similar degrees of incompatibility. We have noted that reliable data for some key element ratios such as

niobium/thorium, niobium/lanthanum and strontium/neodymium are the same in strongly depleted and in fertile spinel-bearing peridotite xenoliths, and this is also true for their garnet-bearing counterparts. These ratios are typical of mid-ocean-ridge basalts and ocean island basalts, but distinct from those observed in island arc basalts^{1,5,6}. So far no garnet- or spinel-bearing peridotite xenoliths have been reported to have the trace-element signatures resembling either those expected of convergent margin basalts and/or their residues. Thus, it is unlikely that significant volumes of the continental lithospheric mantle are developed at convergent plate margins.

Rogers *et al.* argue that the composition of continental basalts can be used to define the composition of the CLM, and that such data indicate CLM growth occurs at convergent plate margins. Other workers, however, continue to argue that many continental basalts may have been significantly affected by crustal contamination (see, for example, refs 5,7). This process would tend to obscure any information that they contain about their CLM source regions. In addition, although the geochemistry of continental basalts is determined partly by the CLM, the 'asthenosphere' is also involved⁸. In characterizing the composition of their source region, therefore, the effects of variable degrees of partial melting, crystal fractionation and the relative contributions of lithospheric and asthenospheric components must be taken into account. Some small-volume magmas (such as kimberlites and ultrapotassics) may be derived from CLM alone⁹, but they, and hence their sources, are volumetrically insignificant. Considering the many variables involved in basalt genesis, it seems unlikely that a unique and unambiguous composition of the continental lithospheric mantle can be deduced from basalt geochemistry. The comments of Rogers *et al.* highlight the critical need for more data on the chemistry of mantle xenolith.

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Cleaning up after Chernobyl

SIR—Confusion still exists regarding the actions that might have been appropriate in the immediate aftermath of the passage of the Chernobyl fall-out cloud over the United Kingdom. Initial reports¹ of the environmental levels and numbers of people exposed to them upon, on which I based an early assessment of the impact on health of the accident² have been revised (J. Simmonds and M. J. Crick, article submitted to *Nature* in March 1987 and subsequently withdrawn) following an article³ and correspondence⁴ in the *National Press*.

Based on the revised values for iodine-131 in milk, I now estimate the average dose to the thyroids of 6-month-old children (the members of the population expected to receive the highest doses from any given level of contamination) drinking fresh cow's milk in the high rainfall areas to have been about 5 mGy (assuming 0.5 litres per day milk consumption), rather than 10–20 mGy (ref. 2). A value of 5 mGy corresponds to about a 10 per cent increase in the spontaneous risk of the largely non-fatal thyroid cancer for those children⁵.

But the measurements reported by the NRPB (National Radiological Protection Board) are described as 'representative'; more recent measurements of caesium-137 on the ground made by aerial survey in Scotland showed local fluctuations of an order of magnitude or more⁶ and cast serious doubt on the validity of deposition estimates based on earlier monitoring and predictions in some upland zones⁶. The possibility that levels of iodine on some pasture might have been 10 or more times higher, and therefore that some local milk supplies might have been more heavily contaminated than the revised NRPB results indicate, cannot now be excluded.

The decision⁷ not to take counter-measures to limit iodine-131 consumption was based on the criteria for limiting exposures of the public in the event of an accidental release of radioactivity, the so-called Emergency Reference Levels⁸. These ERLs are designed to protect individuals from excessive exposure in the early stages of accidents occurring in Britain, and are therefore usually envisaged as being only temporarily applicable to local populations in the vicinity of the accident. Very careful consideration should be given to their extension to the situation where the whole country might be affected and the rapid and comprehensive monitoring of fall-out levels is demonstrably not feasible.

The annual effective dose equivalent limit for members of the public is 1 mSv (ref. 9), whereas the lower ERL of dose applicable to the thyroid is 50 mSv (ref. 8). Thus, after the accident some children will have received about a sixth of the annual dose limit from iodine alone and in

some cases possibly an ERL or more of dose to the thyroid (in this case Sv and Gy are numerically identical). Doses from other sources, including external exposure, could also have been correspondingly greater.

In the case of the short-lived isotopes of iodine it is doubtful whether, in the circumstances of widespread fall-out following the accident, the situation could ever be assessed in time to take effective action, and pre-emptive action, based on early air monitoring data, would be appropriate. The removal of cattle from pasture in the Netherlands for 4 days following the first indication of iodine-131 in milk is reported by NRPB to have saved about 50 per cent of dose to the critical group¹⁰. Experience indicates that, following a release of radioactivity from an operating reactor, the short-lived isotopes of iodine will dictate the immediate response. This response, as advised by the

NRPB^{8,11}, should be based on optimization or on the principle that doses are kept as low as is reasonably achievable, rather than dose limits.

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■ This letter is a revised version of one submitted after the Chernobyl accident in April, 1986.
Editor, *Nature*

Early warning for LTP

SIR—Davies *et al.*¹ recently reported that the induction of long-term potentiation (LTP) is associated with an increase in the sensitivity of hippocampal CA1 neurons to L-glutamate analogues. The increased sensitivity was slow to develop, being first observed 15–30 min after LTP induction, and reaching a peak only after 1–2 hours. The authors interpreted their results as evidence for a presynaptic component mediating the early phase of LTP maintenance. However, postsynaptic sensitivity was assessed in their experiments by monitoring the amplitude of quisqualate (Q) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) potentials which had a very slow time course (compared with the excitatory postsynaptic potentials) and which were generated by very large iontophoretic ejection currents (Q, 46–163 nA for 1–2 s; AMPA, 40–155 nA for 10–25 s). One possibility that should be considered is that the application of such large and prolonged doses of Q and AMPA induced desensitization of the synaptic Q receptors and consequently, the slow iontophoretic responses monitored in the study of Davies *et al.*¹ may have been generated by activation of extrasynaptic receptors. Recent studies have shown that quisqualate and AMPA activate two types of receptors, one rapidly activating and rapidly desensitizing, and the other slowly activating and maintained.

The rapidly activating and desensitizing responses were found in clusters or hot spots, and were suggested to be caused by activation of synaptically lo-

cated receptors, whereas the slowly activating non-desensitizing responses were evenly distributed, and are probably extrasynaptic receptors^{2,3}. If the responses monitored in the study of Davies *et al.* were caused by the activation of extrasynaptic Q receptors, then their location or properties may render them less rapidly responsive to the LTP inducing signal than the synaptic receptors. Thus, early changes in Q synaptic sensitivity following LTP induction may not have been observed.

The time taken to establish an increase in Q postsynaptic sensitivity is important in elucidating the different components of LTP. Thus recent studies have demonstrated that the initial component of LTP is an NMDA receptor induced short-term potentiation (STP), which peaks at 3–5 min and has an overall time course of 10–30 min⁴. If an increase in post-synaptic receptor sensitivity were to occur earlier following the tetanus than that observed by Davies *et al.*¹, then the establishment of LTP could be explained by the initial NMDA induced STP followed immediately by a postsynaptic increase in receptor sensitivity.

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Who made the sky?

William B. McKinnon

Origin and Evolution of Planetary and Satellite Atmospheres. Edited by S.K. Atreya, J.B. Pollack and M.S. Matthews. *University of Arizona Press: 1989. Pp. 881. \$45, £38.95.*

IN the oldest near-eastern mythologies, the world was created in the fertile union of sky and earth. Today, the curtain is drawn partly back, and we ask who or what created the creators. This questioning is exemplified by the latest addition to the University of Arizona's Space Science Series — a volume whose subjects are the origins and fates of the different skies (atmospheres) of our Solar System. The atmospheres are not examined to the exclusion of the solid worlds to which they are intimately coupled (if the solid worlds exist); rather, atmospheres serve as a focus. Atmospheres are, after all, observable: we have also directly sampled the atmospheres of the Earth, Venus and Mars, and are finally on our way (with the launch of Galileo) to sample Jupiter's.

As usual in the Arizona series, this book is based on a conference and, as usual, the text goes far beyond the proceedings. What the reader will find is 22 comprehensive chapters written by 50 carefully chosen experts. Some chapters are simple reviews, others present a wealth of new information. The volume is well-organized into five parts. The first two cover conditions in the early Solar System and so-called 'primitive' bodies (comets, asteroids and their brethren). The other three are devoted to the atmospheres of the terrestrial planets, the deep atmospheres of the giant outer planets, and the satellites of the giant planets. The intended readership is wide, from graduate students to the most senior researcher.

Several early chapters are noteworthy for the non-specialist. Irvine and Knacke and Jessberger *et al.* have put together micro-compendiums on the composition and chemistry of interstellar clouds (from which planets and stars are born) and comets (possibly the least processed stores of interstellar matter), respectively. The literature of interstellar clouds and their gas and dust is vast, and it is most gratifying to have this material collated and synthesized. Somewhat differently, our

knowledge of cometary compositions has been revolutionized by the Halley encounters in 1986, but it is difficult to find a good overview of what comets are now estimated to be made of. A detailed paper on solar nebula chemistry by Prinn and Fegley is not a review at all, but a significant addition to the literature of this topic,



Hidden in a cloud of gas and dust is the nucleus of Halley's comet: a $16 \times 8 \times 8$ km chunk of possibly porous ices, rock mineral grains and organic matter. Such bodies may have been the bringers to the terrestrial planets of air, water and organic precursors to life and may also be eroders of atmospheres and destroyers of climates and ecosystems.

outlining the myriad processes that lead to disequilibrium chemistry during the cooling of the nebula, and thus to new predicted compositions for planetesimals (such as anhydrous silicates and ice rather than hydrated silicates). All three chapters, and several related ones, can be profitably read by anyone interested in the composition of any part of any planet or satellite.

One clear message delivered in this book is how far we have come in understanding the climatic evolution on the terrestrial triad — Venus, Earth and Mars. W.W. Rubey's original conception of an Earth accreted cold and then slowly releasing its atmosphere and ocean because of volcanism has been swept away. The planets accrete hot, and any world larger than Mars-sized is melted and degassed. The atmospheres so created are subject to further additions or losses due to impacts (Ahrens *et al.*). Losses also occur via thermal diffusion, solar wind stripping and hydrodynamic blow-off (Hunten *et al.*). Volatile gases and water can recombine with the planetary interior by dissolving in global magma oceans or by

quieter chemical recombination. Climates are then subject to marvelous feedbacks between the interior and atmosphere (Kasting and Toon; Schubert *et al.*). That the Earth has been habitable for most of its history is a central focus of the chapter by Kasting and Toon, and it is remarkable that a story can be told at all. Much work remains to be done, but the authors, in arguing that an Earth-like planet orbiting a solar-type star can maintain surface liquid water at orbital distances of about 0.95 to about 1.5 astronomical units (the orbit of Mars), have tantalizingly extended their conclusions to other solar systems.

Important constraints on the origins of terrestrial planet atmospheres are provided by noble-gas inventories and their isotopic compositions (dealt with in several chapters). For example, these may imply that the Earth and other terrestrial planets accreted within the roughly 1–10 million-year lifetime of the solar nebula, and not over a much longer span of time, as has been generally thought. It is also clear from the chapter by Lunine *et al.* that similar information will be crucial for understanding the formation and evolution of Titan's thick, nitrogen-methane atmosphere. This information should be provided by

the Titan probe on board the planned Cassini mission. (It is a shame that Ganymede and Callisto around Jupiter do not possess atmospheres. What stories they could tell us!) It is also thought-provoking to read this chapter with the new knowledge of Triton's nitrogen-methane atmosphere and the evidence for a 'heavy' gas, such as carbon dioxide or nitrogen, around Pluto.

It is truly exciting to read through this volume. The Arizona series traditionally focuses on individual bodies or classes, but the challenge here of thinking about the histories of the atmospheres of such diverse worlds as the Earth, Jupiter and Triton, has led many of the authors to broader, synergistic views and thinking. The reader will be similarly enticed. This book naturally belongs on every planetary scientist's bookshelf, and because of its broad sweep, should find a ready home among astronomers and geoscientists as well. □

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Shaking the very foundations

Warwick Bray

Debating Archaeology. By Lewis R. Binford. *Academic*: 1989. Pp. 534. \$44.95, £28.50.

EVERY few years Lewis Binford reissues a collection of his essays, a form of intellectual autobiography that invites the reviewer to assess the author's personality and significance within the profession, as well as his views on particular archaeological subjects. In the case of Binford, who has done little to discourage the cult of personality, it is almost impossible to separate the man from his works. To his many admirers he is the greatest archaeological thinker of his generation and a true prophet; others regard him as archaeology's equivalent of a television evangelist. On the evidence of the present book, both these views are exaggerated.

The papers included here have been published in various journals between 1983 and 1989. The first group deals with Binford's familiar themes — what archaeology is for, and how it should be carried out. In his usual trenchant style he sets about his old enemies, the post-processualists and the empiricists, and, eventually, attacks just about every new idea since the 1970s which he did not think up himself.

These chapters are full of robust common sense and, as an exercise in debunking theoretical pretension, are a delight to read. But, one is entitled to ask, is Binford's own philosophy of archaeology any more convincing than those he attacks? Since the 1960s he has asked important but awkward questions, and has done more than anyone to improve data handling by insisting that things are made objective, quantifiable and testable, as far as these ideals can ever be realized in archaeology. In the end, though, Binford (like most of his opponents) is preaching a personal faith. When he says (p. 115) that "the practical limitations of our knowledge of the past are not inherent in the nature of the archaeological record" but are due to "our methodological naivete", this is simply a statement of faith, a personal opinion masquerading as an axiom, and I, for one, do not believe it. By all means let us improve out methodology, but I doubt that this will somehow make all things knowable.

Similarly, Binford's insistence that archaeology is a science, repeated again and again throughout the book, may give the subject a spurious respectability, but is untrue. Science does not have a monopoly on logic, and the fact that archaeologists draw on scientific evidence (as a judge

might listen to a forensic specialist) does not in itself make archaeology a science. This kind of statement ignores the whole purpose of the investigation, and reduces archaeology to a largely descriptive discipline, one which has no way of tackling problems of causality (the traditional preserve of philosophers, historians and theologians) and, indeed, one which has no legitimate interest in such things.

This attitude explains the preoccupation of the 'new archaeology' in the 1960s with the search for laws of human behaviour, analogous to 'scientific' laws governing, say, the behaviour of gasses. Strangely, the failure to find such laws has done little to destroy the myth of archaeology as science. At a deeper level of analysis, as neither philosophers nor scientists can agree about why human cultures have developed as they have, there is no reason to expect a consensus among archaeologists. Nevertheless, at times the debate seems more like a power-struggle between competing prophets than a genuine search for truth. What I object to most of all is the way in which each theoretician insists that his is the only way to do archaeology. This is nonsense, and is intellectual totalitarianism of the worst kind.

Although the early chapters of this book will provoke and stimulate anyone with an archaeological conscience, the later ones concern more specialized matters: ethnoarchaeological studies of stone-quarrying and tool-manufacture by Australian aborigines; butchering practices among the Nunamiut Eskimos; and the taphonomy of Pleistocene bone assemblages and their significance for reconstructing early human behaviour. Binford here applies his own methodological principles to real archaeological problems, with an awareness that almost any case study can be the starting point for a discussion of wider issues.

A quick check among my colleagues suggests that Binford's conclusions are not widely accepted by Pleistocene specialists, but in a book called *Debating Archaeology* the questions matter more than the answers. In future histories of archaeology, Binford may not figure in the list of those who solved the great archaeological problems, but he certainly deserves a place as a teacher and polemicist, a person who has forced us all to think. □

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New in paperback

■ *Theology and the Scientific Imagination: from the middle ages to the seventeenth century* by A. Funkenstein, published in 1986, is now available in paperback. The author attempts to define the points of transition from mediaeval to early modern modes of reasoning. Published by Princeton University Press, price \$14.50.

Emerging from the deep

P.J. Butler

Diverse Divers: Physiology and Behavior. By G. L. Kooyman. *Springer-Verlag*: 1989. Pp. 200. DM 178, £63.50, \$99.

THOSE reptiles, birds and mammals that spend much of their time submerged under water are often in the public eye. Conservation of whales and marine turtles, viral infections of seals and the effect of oil spillages on all marine life — especially, perhaps, birds — has put these animals firmly in the news. Largely from the observation of the early whalers, the ability of some of these air-breathing vertebrates to remain under water for impressively long durations has been studied for more than a century. Only relatively recently has it been possible for the behaviour and physiology of these fascinating animals to be investigated together. One of the pioneers of such studies and an undoubted world expert on the subject is the author of *Diverse Divers*. Nevertheless, because of the detailed nature of the book, I doubt that it will be read by many laymen.

The book's theme is how the anatomy and physiology of animals sets the limits of their behaviour and thus of their exploitation of the sea. As such, I found it frustrating that the animals' behaviour is dealt with at the end — this is certainly a case where I strongly recommend that the reader begins with the last chapter to put the rest of the book into its rightful context.

The main problem facing diving vertebrates is the management of the body's oxygen stores during the period of submersion. Kooyman covers all the factors affecting the size of these stores and the rate at which they are used. The clear conclusion is that, contrary to the traditional view, the vast majority of underwater activity, be it feeding, exploration or merely moving from one place to another, is fuelled by aerobic metabolism. Although perfectly logical, this is nonetheless remarkable in an animal such as the female northern elephant seal which, after weaning her pup, spends almost 90 per cent of her time under water at depths usually in excess of 200 metres. Kooyman emphasizes the very pertinent point made by Kramer in 1988, that animals such as these should be called surfacers and not divers.

Kooyman's first-hand experience of working in the field with the subjects of the book is apparent from the enthusiastic way in which he writes. Unfortunately, many of his stated objectives are not always achieved, and there are several

examples of misquotations and carelessness. When discussing the "flaws" and "weaknesses" in the conclusions of others, it is necessary to be aware of the full extent of these conclusions. On p. 72, Kooyman quotes data from a paper of mine published in 1983, but refers to a conclusion from one published four years earlier. Reference to any paper that I published after 1983 will show that my current view agrees with his own. Silly errors like stating that "available O₂ store is constant for any size" [of seal] (p. 148) and that the effect of temperature on the oxygen equilibrium curve for haemoglobin is part of the Bohr effect (p. 110) detract from the overall impression of competence.

Perhaps Kooyman had a limited amount of time between field trips to write this monograph yet, true to his determined nature, he did it. The result of this determination is that the finished product is not as polished and factually reliable as it might have been. It is, nevertheless, a personal perspective by a scientist who is highly regarded by his peers, and the basic philosophy and coverage of the available data are commendable. This monograph will be seen as a landmark in the unfolding story of how some of the tetrapod vertebrates have

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

— anatomy and physiology set the limits of
managed successfully to colonize the
aquatic environment. □

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Return to obscurity

H. S. Yoder Jr

Boninites and Related Rocks. Edited by A. J. Crawford. *Unwin Hyman: 1989. Pp. 465. £60, \$90.*

Carbonatites; Genesis and Evolution. Edited by Keith Bell. *Unwin Hyman: 1989. Pp. 618. £85, \$125.*

TWO rare rock-types, boninite and carbonatite, are here discussed by two separate groups of current investigators. The boninite compilation is a new effort, whereas the carbonatite review, containing critical new data, records the advances made in the 25 years since publication of *Carbonatites*, the first volume on the topic. The new review retains only three of the authors from the earlier work. Both of the new books will generate substantial debate.

The attempt to make boninite, an obscure rock type, a primary magma fails. Readers will be alerted immediately to the editor's problem by comparing the title

on the spine ("Boninites and Related Rocks") with that on the title page ("Boninites"). The editor has tried to bring together several papers on boninites, some already in preparation before this volume was planned. But most of the rocks from various localities described by the authors do not have the characteristics of boninite as originally described from the type locality, the Bonin Islands.

After the introductory chapter formalizing the term 'boninite', the use of which had lain fallow for more than 80 years, the authors of the subsequent chapters dutifully point out similar characteristics in rocks which they call high-magnesium andesites, bronzite andesites, basaltic andesites, bajaites, sanukitoids, lamproites, high-magnesian basalts and high-magnesian tholeiites. A reluctance to use the term boninite is evident, the rocks under consideration being at most referred to as boninitic, boninite-like or having boninite affinities.

The case for reviving the suggestion that boninite is a primary magma type rests on rocks that have often been metamorphosed to greenschist facies, altered by rock-water interaction or weathered. Large amounts of new analytical data are

presented here, but no norms are given in the rock-composition tables. The author of one paper, however, notes a range from olivine to quartz normative composition, and the authors of three others use a molecular norm plot. In fact, if the reader calculates the norms of the unweathered, unmetamorphosed rocks within the specified silica limits, it will be seen that they are quartz normative in the range of most andesites. The large amount of clinopyroxene in the norms raise questions about their derivation from harzburgite, which rarely contains clinopyroxene. Reasons for the absence of modal plagioclase are not discussed even though the normative contents of feldspar, had they been presented, in listed 'representative' rocks range from 25 to 67 weight per cent. Furthermore, the rarity of amphibole is not explained even though the water content of the magma is estimated to be from one per cent to oversaturated.

A principal problem dealt with in this book is the paradox between the high-magnesium content and the alkali- and light-rare-earth-element enrichment of boninite. Most of the authors support the party line favouring the derivation of the magma from a depleted source enriched by two or three separate events. Some suggestions for this enrichment include magma mixing, assimilation of subducted crust, alteration by fluids from slab dehydration, mantle metasomatism or contamination by wedge material. This melange of hypotheses is supported primarily by interpretations of trace elements from the same groups of metamorphosed, altered and weathered rocks. The various "scenarios" for boninite magma generation in the plate-tectonic environment will no doubt entertain tectonists, and the two-element plots and spider diagrams will capture the attention of geochemists. But the fact that most of the plots of major elements of the 'related' rocks are splatter diagrams will not win many converts to the concept of an orderly 'boninite spectrum'.

This collection of 16 papers would have benefited considerably had they been presented at a symposium and been subjected to external review before publication as a special issue of a journal. Had this been the case, there would have been the opportunity for reader discussion and rebuttal.

By sharp contrast, the reappraisal of carbonatites is a mature effort, seasoned by symposium discussion. The 23 papers record lively debates on the very existence of carbonatite as a primary magma; the source and nature of volatiles; tectonic environment preferences; sequencing and periodicity; trace-element partitioning; diagnostics of magmatic heritage; and the importance of association with silica-rich rocks.

After several general chapters on chem-

ical compositions, spatial and temporal distributions, and field relations, separate chapters are devoted to the classic localities such as those at and around Kaiserstuhl, Phalaborwa, Ice River, Arusha and Oldoinyo Lengai. The well-known arguments of origin via immiscibility versus fractionation versus direct partial melting; melt versus fluid metasomatism as related to fenitization; and the iron and alkali enrichment versus depletion schemes are displayed with blunt force. Fortunately, new experimental data are given that help to resolve some of these issues, but even

the experimentalists stand their separate ground on details. Whether there are direct or indirect links of carbonatites to kimberlites, nephelinites, melilitites and phonolites is another issue not taken lightly. Because of the economic importance of many elements in carbonatites, the debate will no doubt increase as new data are acquired. Keep your score card handy. □

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Hybrid mystery

Mark Ridley

Speciation and Its Consequences. Edited by Daniel Otte and John A. Endler. *Sinauer Associates, Sunderland, Massachusetts: 1989. Pp. 679. Hbk \$50, £40; pbk \$29.95, £23.95. Distributed in Britain by W.H. Freeman.*

JOHN Herschel, in a grand phrase that Darwin knew well, described the question of how new species arise as "the mystery of mysteries". The mystery has now been cut down to size. We know the general mechanisms by which it must take place; but the exact circumstances and processes of speciation still remain rather too mysterious, given that it is probably the most researched area of evolutionary biology. It is characteristic that a multi-author book on the subject should contain not only a suggestion (by Coyne and Orr) that the biological species concept (see below) and allopatric theory of speciation are "the only two real advances in our understanding of speciation", but also several persuasive criticisms of the biological species concept including one (by Lynch) which concludes, from evidence that cannot just be brushed aside, that "peripheral isolates speciation" (the most popular allopatric model) "may be even rarer than sympatric speciation among vertebrates".

Lynch supports his conclusion with a combination of biogeographical and phylogenetic evidence. He has picked several vertebrate taxa whose phylogenies are well known and looked at the geographical ranges of closely related (sister) species pairs. He classifies the pairs according to whether their ranges are substantially overlapping (suggestive of sympatric speciation), non-overlapping with one much smaller than the other (peripheral isolate speciation), or have little overlap and similar sizes (vicariance — either allopatric speciation by subdivision, or parapatric speciation). For a sample of 66 speciation events, "the vicariant model explains 71%, the peripheral

isolates model initially explains 15%, and sympatric speciation explains 6%".

Reinforcement has now emerged as a question almost as big as geography. The idea is that if two populations have evolved some way towards speciation, hybrids between them may be disadvantaged, and selection will favour assortative mating within each population (each is selected to mate only with its own type, to avoid producing hybrids). It is an old idea, dating back to Wallace, and has been more or less taken for granted in the literature. The theory seemed sound, it had been illustrated by artificial selection experiments, and it explained various lines of field evidence. But in the past ten years, this has all been turned on its head. In his chapter, Butlin reviews the developments. The theory has been so undermined that he can remark: "since theory suggests that reinforcement is improbable, the evidence must be very strong before a putative example can be accepted". He explodes the artificial selection experiments and finds all sorts of alternatives to reinforcement in the field evidence, leaving the conclusion that reproductive isolation only ever evolves as a consequence of other evolutionary changes. I feel rather reactionary about these developments. It is one thing to pick holes in an interpretation, but another to show it is wrong; reinforcement still seems to me the most natural explanation of such phenomena as character displacement and artificially selective reproductive divergence. The counter-theory, moreover, presupposes either unstable polymorphisms or special genetic models of choice that may not be the only possibilities. But reinforcement has at least been turned back into something to be argued for, and not taken for granted.

Coyne and Orr show, from comparative data and ingenious breeding experiments in the fruitfly *Drosophila*, that the early stages of speciation result from changes in X-linked genes, and divergence at genes on other chromosomes follow. They explain this tendency by the more rapid evolution of recessive genes on the sex chromosomes than on autosomes (where recessive genes are less often expressed).

The difference between sex chromosomes and autosomes does not exist for dominant genes, and Coyne and Orr conclude that speciation is genetically peculiar, being initiated particularly by recessive genes.

Templeton and Cracraft both criticize Mayr's biological species concept in a manner that I believe distorts the concept in a subtle but crucial manner. Mayr defined a species as a group of "interbreeding populations reproductively isolated from other such groups". The definition mentions two properties: interbreeding and reproductive isolation. Templeton (following H.E.H. Paterson) renames this the "isolation" species concept and treats it as if it defined species only in terms of evolved isolation mechanisms. For example, it is possible that two recently diverged populations, living in separate geographical areas, could form two interbreeding groups, even though there was no evolved isolating mechanism between them (such as a difference in reproductive behaviour). Templeton produces this case as a damaging counter-example to Mayr's definition, and a reason to prefer a concept that defines a species by interbreeding, regardless of whether it possesses any isolating mechanisms. The argument, however, hypostatizes one part of Mayr's definition and ignores the other. Mayr defined species by interbreeding too — he put it first, before reproductive isolation. Moreover, the "reproductively isolated" in Mayr's definition need not mean an evolved isolating mechanism. The allopatric populations of Templeton's would-be counter-example do satisfy Mayr's definition: they are reproductively isolated, even if only by a geographical barrier.

There is more to the dispute, however. Templeton argues for a "cohesion species concept" to include asexual as well as sexual species; here there is a genuine disagreement. And some statements of the biological species concept have explicitly mentioned isolation mechanisms, rather than mere reproductive isolation. But the biological species concept has always explained species integrity by gene flow, and to oppose it on the grounds that it is an "isolation" rather than an "interbreeding" species concept is, I believe, bogus.

The 25 chapters comprising the book are all by experts, and (with, to my taste, only two exceptions) attain a consistently high standard. They cover all research methods — population and ecological genetics, systematics and ecology: only the palaeobiologists are left out, but even they should find as much to interest them here as the rest of us. □

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A regulatory hierarchy for cell specialization in yeast

Ira Herskowitz

The specialized sets of genes that determine different cell types in yeast are controlled by combinations of DNA-binding proteins some of which are present only in certain cell types whereas others are present in all cell types. Final differentiation requires an inductive signal that triggers both gene transcription and cell-cycle arrest. Synthesis of the proteins coded by the 'master regulatory' mating-type locus is regulated so as to generate a heterogeneous mitotic cell population containing a stem-cell lineage.

ALTHOUGH budding yeast is a unicellular organism, it exhibits many of the processes that are crucial to the development of multicellular organisms, such as cell specialization and cell-cell interactions. The relative simplicity of yeast and its experimental tractability have made it possible to synthesize an integrated view of how cell specialization in yeast results. This view encompasses transcriptional regulatory proteins as well as response to intercellular growth and differentiation factors.

In a multicellular organism, the different types of specialized cell play different roles in the intact organism. In yeast, the specialized cells play different roles during the life cycle of the organism¹⁻³. There are three different types of yeast cell: the **a** and α mating types are typically haploid and are specialized for mating with each other. Mating entails the fusion of the two haploid cells to produce the third cell type, the **a**/ α diploid cell, which can undergo meiosis and sporulation when nutritionally starved.

There are many differences among these cell types. Perhaps most notably, the **a** and α cells each have a distinctive cell-signalling system that enables mating to take place between the two partners. The α cells produce an extracellular peptide, α -factor, which acts on a receptor produced only by **a** cells; **a** cells likewise produce an extracellular peptide, **a**-factor, that acts on a receptor produced only by α cells. **a**/ α cells are incompetent for mating and neither produce nor respond to either factor.

All of the differences between these cell types are determined by the two alleles of the mating-type locus, *MAT*: cells with the *MATa* allele exhibit the **a** mating type, cells with the *MAT α* allele exhibit the α mating type, and cells with both *MAT* alleles exhibit the **a**/ α phenotype. These alleles turn out to encode components of regulatory proteins that occupy a key position in yeast cell specialization: they govern expression of specialized gene sets and sit at the top of the regulatory hierarchy that generates a specific mitotic cell lineage.

I begin with an explanation of what we know about how the products of the **a** and α alleles determine yeast cell types. We shall see that these products do not act alone and shall identify their molecular co-participants and their regulators. Furthermore, we shall see that cell specialization results from a combination of cell-autonomous programming (by these regulatory proteins) and environmental response (induction), two recurring themes in classical developmental biology. The molecular information obtained from studies of yeast may be directly applicable to understanding the parts played by these processes in development of multicellular eukaryotes and how different combinations of transcriptional regulators can give rise to distinctive cell types.

Differential gene expression

Four classes of genes are defined on the basis of their distinct patterns of expression in the three yeast cell types (Table 1). α -specific genes are expressed only in α cells; **a**-specific genes are expressed only in **a** cells; haploid-specific genes are

expressed in both haploid cell types but not in diploid (**a**/ α) cells; and sporulation-specific genes are expressed only in **a**/ α diploid cells (and then only after nutritional starvation). These differential patterns of expression are directly determined by the products of the *MAT* locus (except for sporulation-specific genes; discussed below).

The *MAT* locus codes for three polypeptides^{4,5} that are components of regulatory proteins: **a1** is encoded by the *MATa* allele and is thus produced only in **a** cells; $\alpha1$ and $\alpha2$ are encoded by the *MAT α* allele and are produced only by α cells. These products are responsible for three regulatory activities⁴: $\alpha1$, $\alpha2$ and **a1** - $\alpha2$. Figure 1 shows how these three activities programme expression of the four classes of genes. $\alpha1$ is a positive regulator of transcription of the α -specific genes—it is needed for their expression. $\alpha2$ is a negative regulator of transcription of the **a**-specific genes—it blocks transcription of these genes. Thus, in an α cell, the α -specific genes are expressed whereas **a**-specific genes are not; hence, the cell displays an α phenotype. The **a1** protein produced in an **a** cell does not play any role in programming the **a** cell phenotype. Rather, the appropriate gene set (**a**-specific genes) is expressed because of the absence of the negative regulator $\alpha2$, and the inappropriate gene set (α -specific genes) is not expressed because of the absence of the positive regulator $\alpha1$. **a1** does have an important role in **a**/ α cells, where it associates with $\alpha2$ (ref. 6; C. Goutte and A. Johnson, personal communication) to comprise a novel negative regulatory protein, **a1** - $\alpha2$, that is responsible for many

TABLE 1 Cell-type-specific gene sets

Gene set	Representative members	Cell type		
		a	α	a / α
a -specific genes	<i>STE2, STE6, BAR1</i>	+	-	-
α -specific genes	<i>STE3</i>	-	+	-
Haploid-specific genes	<i>STE12, HO, RME1</i>	+	+	-
Sporulation-specific genes	<i>IME1, SPO13</i>	-	-	+

a-specific genes include those coding for the structural genes for **a**-factor (*MFA1* and *MFA2*) and a protein necessary for its secretion (*STE6*)¹⁰⁵, the receptor to α -factor (*STE2*), and a protease that degrades α -factor (*BAR1*). α -specific genes include those coding for the structural genes for α -factor (*MFA1* and *MFA2*) and the receptor to **a**-factor (*STE3*). Haploid-specific genes include those necessary for mating (*STE4, STE5, STE12* and *STE18*), nuclear fusion (*KAR1*), mating-type interconversion (*HO*), regulation of meiosis (*RME1*), and also for transcription of the retrotransposon *Ty1* (refs 36, 106, 107). The genes coding for subunits of the G protein—*SCG1/GPA1, STE4* and *STE18* (ref. 108)—are all haploid-specific genes. Sporulation-specific genes are expressed only after nutritional starvation and include an activator of early events in meiosis (*IME1*; ref. 21), genes necessary for proper meiotic chromosome segregation (*SPO12* and *SPO13*), and the sporulation-specific glucoamylase¹⁰⁹. Additional references can be found in refs 1 and 2.

of the properties of this cell type. In particular, $\alpha 1$ - $\alpha 2$ turns off synthesis of $\alpha 1$ (refs 7, 8), which eliminates transcription of the α -specific genes. $\alpha 1$ - $\alpha 2$ also turns off expression of the large group of haploid-specific genes, which are otherwise expressed in both haploid cell types.

Our understanding of control of yeast cell type has come from a wealth of *in vivo* and *in vitro* observations. First of all, mutants defective in the different products of *MAT* exhibit defects in regulation of the target gene sets: for example, α cells that lack $\alpha 1$ fail to express α -specific genes⁹; mutants that lack $\alpha 2$ show the inappropriate behaviour of expressing α -specific genes¹⁰; mutants defective in either $\alpha 1$ or $\alpha 2$ are unable to turn off haploid-specific genes^{11,12}. Specific DNA sequences located in the upstream regions of the different gene sets have been identified that confer cell-type-specific expression on reporter genes¹³⁻¹⁶. Finally, the polypeptides encoded by *MAT* have been shown to be components of site-specific DNA-binding proteins which bind to upstream regulatory regions of their target genes^{6,15,17-19}. These studies show that the proteins that bind to the specialized genes *in vitro* are responsible for regulation of these genes *in vivo*.

The sporulation-specific genes differ from the other classes in that they are not directly regulated by the products of the *MAT* locus. $\alpha 1$ - $\alpha 2$ does not bind directly to the sporulation-specific genes, but activates them by turning off synthesis of an inhibitor of their expression^{12,20}. This inhibitor is the product of the *RME1* gene, which blocks an early step in meiosis²¹ (see Fig. 1).

Mechanisms of activation and repression

$\alpha 1$ and $\alpha 2$. The products of the *MAT* locus do not act on their own to produce the cell-type-specific pattern of gene expression in haploid cells, but interact with a common protein (originally termed PRTF¹⁸ or GRM¹⁷) that is present in all cell types. The alphabet-soup of different names for the same factor has been simplified (or made more complex, depending on one's outlook) by the recent finding that PRTF/GRM is encoded by the *MCM1* gene²²⁻²⁴. I shall refer to it hereafter as MCM1. This protein has binding sites in the upstream regulatory regions of both the α -specific and the α -specific genes¹⁷⁻¹⁹ and seems to be a transcriptional activator of both gene sets^{13,17,25,26}. It is the $\alpha 1$ and $\alpha 2$ products of *MAT* that determine whether transcriptional activation takes place (Fig. 2). Research on how they do this has revealed molecular mechanisms that are likely to underlie other examples of eukaryotic cell specialization.

In α cells, MCM1 is able to bind to its site in the upstream region of α -specific genes and is thus able to stimulate transcription. MCM1 cannot bind to its targets in α -specific genes to activate these genes (for reasons explained below). Thus, in α cells, α -specific genes are expressed and α -specific genes are not.

In α cells, MCM1 must be helped to activate the α -specific genes and prevented from activating the α -specific genes. These are functions of $\alpha 1$ and $\alpha 2$ proteins, respectively (see Fig. 2). $\alpha 1$ might assist MCM1 in two ways. First of all, $\alpha 1$ (which binds to its target site only in the presence of MCM1) enhances binding of MCM1 to its binding site (the 'P' site)^{13,18,19,23}. Although this might be the sole role of $\alpha 1$, studies *in vitro* of binding of $\alpha 1$ and MCM1 to P sites have led Tan *et al.*¹⁹ to raise an additional possibility. They suggest that MCM1 has different conformations depending on the site to which it binds. When it binds to the P site in α -specific genes, it is in the active conformation for stimulating transcription. By contrast, binding of MCM1 to a P site in an α -specific gene does not induce the active conformation; this results only when $\alpha 1$ binds adjacent to it. Whatever $\alpha 1$ is doing, an elegant and informative analysis by Sprague and colleagues^{13,18} shows that it is MCM1, and not $\alpha 1$, that is the activator. A synthetic P site ('P-PAL') was constructed that is a perfect palindromic version of naturally occurring P sites from different α -specific genes. This site allows binding of MCM1 *in vitro* without $\alpha 1$ and, when used as an

upstream activating sequence for a reporter gene, it allows expression in all cell types—in other words, in the absence of $\alpha 1$. These observations indicate the MCM1 is responsible for activating α -specific genes and that $\alpha 1$ merely assists MCM1.

As noted by Jarvis *et al.*²⁴, control of α -specific genes by MCM1 and $\alpha 1$ provides a way to think about how cell-type-specific regulation might evolve from a constitutively expressed gene. In the first step, the binding site for a general transcriptional activator (analogous to MCM1) becomes enfeebled by mutation. The reduced binding ability is subsequently compensated for by a second protein (analogous to $\alpha 1$), which is present in some cell types but not in others. It is also possible to imagine that activator proteins such as the oestrogen receptor could adopt helper proteins to assist them in binding to weak, imperfect-palindromic sites²⁷.

How does MCM1 stimulate transcription? MCM1 contains an acidic region (ref. 26; see also refs 23, 24) and thus may stimulate transcription in the same way as proposed for other activator proteins containing an 'acid blob', by direct contact with a component of the transcription machinery²⁸. The nucleotide sequence of *MCM1* also shows that it is similar to a protein that is thought to be a transcriptional regulator in mammalian cells, the serum response factor (SRF), which binds to the upstream region of the *fos* gene²⁹; moreover, MCM1 is able to bind to the SRF binding site *in vitro*^{23,30}, suggesting strong conservation of DNA-binding motifs in phylogeny. The functional consequences of the binding of MCM1 to its natural sites

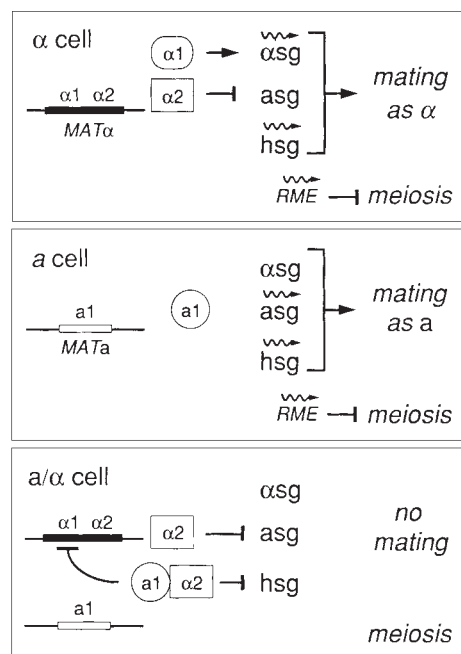


FIG. 1 Expression of cell-type-specific gene sets is governed by regulatory proteins coded by the mating-type locus. The three panels show how the regulatory proteins coded by the mating-type locus ($\alpha 1$, $\alpha 2$ and $\alpha 1$ - $\alpha 2$) govern transcription of three different gene sets (α sg, α -specific genes; α sg, α -specific genes; and α sg, haploid-specific genes). Transcription is indicated by a wavy arrow. $\alpha 1$ activates transcription of α -specific genes; $\alpha 2$ inhibits transcription of α -specific genes; $\alpha 1$ - $\alpha 2$ inhibits transcription of haploid-specific genes. *RME1* is a haploid-specific gene whose product inhibits initiation of meiosis (probably by repression of the *IME1* gene; ref. 21) and thus expression of the *ssg* (sporulation-specific) gene set. As a result of action of these regulatory proteins, the three cell types exhibit the indicated phenotypes with respect to mating and meiosis. Members of the different gene sets are listed in Table 1. Nucleotide sequences of *MAT α* and *MAT α* are given in ref. 5. *MAT α* contains 747 bp of DNA (drawn as a black rectangle) that is not present in *MAT α* ; *MAT α* contains 642 bp of DNA (drawn as an open rectangle) that is not present in *MAT α* .

in yeast cells, however, are profoundly influenced by both the DNA and the cellular context of the binding, in a way that is likely to be true of many DNA-binding proteins in specialized eukaryotic cells.

Thus, in the upstream regions of **a**-specific genes, there are $\alpha 2$ binding sites on both sides of the binding site for MCM1; $\alpha 2$ binds to these sites to prevent MCM1 from activating the transcription of **a**-specific genes. There are two ways in which $\alpha 2$ might repress transcription¹⁷. $\alpha 2$ may 'mask' MCM1 (for example, by covering up its activation domain) so that it cannot make contact with other parts of the transcription machinery. Another possibility is that the MCM1- $\alpha 2$ complex is able to contact the transcription machinery, but 'locks' it in a nonproductive mode. The masking explanation is similar to that invoked for the yeast negative regulator, GAL80, which binds to the activator GAL4, and is thought to cover up the GAL4 activation domain³¹. Although the masking model for $\alpha 2$ is appealing, it does not explain how MCM1- $\alpha 2$ can repress transcription when it acts upstream of an intact transcriptional activation region¹⁵.

$\alpha 2$ (and $\alpha 1$) contain a segment that is similar to the homeodomain found originally in *Drosophila* proteins^{32,33} and that has since been discovered in other families of DNA-binding proteins³⁴. Keleher *et al.*¹⁷ suggest that the properties of $\alpha 2$ may provide an answer to how these proteins, which have relatively low specificity of binding, recognize their target sites. Just as MCM1 increases the affinity of $\alpha 2$ for its operator at least 50-fold¹⁷, an MCM1 analogue might enhance the ability of homeodomain proteins to recognize their target sites. Perhaps the conserved region adjacent to the homeodomain of the POU family of proteins³⁴ contacts such a companion protein.

$\alpha 1$ - $\alpha 2$. $\alpha 2$ is versatile: in association with MCM1, it represses transcription of **a**-specific genes; but in conjunction with $\alpha 1$ it represses the haploid-specific genes. This regulatory species, **$\alpha 1$ - $\alpha 2$** , is present only in **a**/ α cells and is a molecular signature for this cell type. In the presence of $\alpha 1$ polypeptide, $\alpha 2$ exhibits a new binding specificity⁶, recognizing the **$\alpha 1$ - $\alpha 2$** operator sequence^{16,35}, which does not contain an MCM1 binding site (C. Goutte and A. Johnson, personal communication; and ref. 6). Although one might have imagined that all $\alpha 2$ subunits in an **a**/ α cell would be present as the heteromeric **$\alpha 1$ - $\alpha 2$** species, this is not the case⁶: **a**/ α cells contain both species of repressor, $\alpha 2$ and **$\alpha 1$ - $\alpha 2$** , which recognize distinctive operator sites to turn off two different gene sets (the **a**- and haploid-specific genes).

It is likely that **$\alpha 1$ - $\alpha 2$** works by preventing other proteins, such as transcriptional activators, from binding to DNA. Genes repressed by **$\alpha 1$ - $\alpha 2$** comprise a diverse collection that uses different activator proteins. For example, **$\alpha 1$ - $\alpha 2$** blocks transcription of the Ty1 element³⁶ and the *HO* gene^{11,16}, which require different transcriptional activators^{37,38}.

Combinatorial control. Molecular analysis of the specialized cell types of yeast demonstrates the way in which combinations of polypeptide subunits generate different regulatory activities. Table 2a shows the distribution of subunits in the three different cell types. In addition to listing **$\alpha 1$** , $\alpha 2$ and MCM1, I include the STE12 protein, which is discussed below. These subunits interact with each other in a pairwise way to generate several distinctive regulatory species (Table 2b): **$\alpha 1$ -MCM1**, **$\alpha 2$ -MCM1**, **$\alpha 1$ - $\alpha 2$** and **MCM1-STE12**. A comparison of the subunits (Table 2a) with the regulatory activities resulting from their pairwise association (Table 2b) shows clearly how combinations of different subunits (such as members of the POU family) can generate cell-specific gene expression³⁴.

Induction of differentiation

Although **a** and α cells in pure culture exhibit many of their characteristic cell specializations, such as production of mating factors and receptors, some specializations are seen only when **a** and α cells are cultured together. A particularly striking example of this is expression of the *FUS1* gene, the product of

which is necessary for cell fusion during mating. Its synthesis is induced at least 500-fold (refs 39, 40). In the presence of cells of opposite mating type, synthesis of the mating factors and receptors—in fact, all of the **a**-specific and α -specific genes that have been tested—is also induced, in this case roughly 3- to 5-fold (refs 25, 41, 42; S. Michaelis, K. Kubo and I.H., unpublished observations; summarized in ref. 2). It is the mating factors that are responsible for this induction. α -factor, a peptide of 13 amino acids, is secreted from cells and is freely diffusible. **a**-factor is a peptide of 12 amino acids whose last residue is a farnesylated cysteine that also contains a methyl ester⁴³. Although **a**-factor is found extracellularly⁴⁴, it is conceivable that **a**-factor is anchored to the **a** cell membrane by the farnesyl group and is presented to α cells by cell-cell contact rather than by diffusion.

The mating factors have several additional effects on responding cells (see ref. 2), all of which serve to coordinate and facilitate mating. This induction can be viewed as the final differentiation of a yeast cell, which occurs only when the mating partner is present. Another effect of the mating factors is that they induce responding cells to arrest cell division in G1 (refs 44, 45). Thus **a**-factor and α -factor are negative growth factors. They may work by interfering with the activity of the CDC28 protein

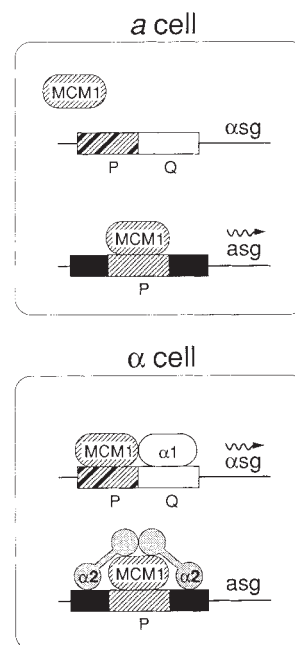


FIG. 2 Transcriptional control of α - and **a**-specific genes by $\alpha 1$, $\alpha 2$ and MCM1 (aka PRTE, GRM). Appropriate expression of α -specific and **a**-specific genes in an **a** cell (upper panel) and in an α cell (lower panel) is mediated by interactions of $\alpha 1$ and $\alpha 2$ with MCM1 protein in the upstream regulatory regions of the target genes. The MCM1 binding site is labelled 'P' and the $\alpha 1$ binding site 'Q'. The P sites are drawn differently for α - and **a**-specific genes to indicate that MCM1 does not bind in the same way to both. In the **a** cell, MCM1 binds to its site in the upstream region of the **a**-specific genes and stimulates their transcription. It is unable to bind effectively to the P site in the upstream region of α -specific genes. In the α cell, $\alpha 2$ binds next to MCM1 in the upstream region of *asg* and thereby represses their transcription. $\alpha 1$ helps MCM1 to activate transcription of α -specific genes by assisting its binding or by inducing it to adopt a conformation suitable for activating transcription. $\alpha 2$ binds as a dimer to the sites drawn in black on opposite faces of the double helix^{17,114}. Each $\alpha 2$ monomer is composed of two domains¹¹⁴: the C-terminal domains bind to DNA, and the N-terminal domains contact each other and MCM1 protein. MCM1 is inferred to be a dimer. The diagram is not drawn to scale: an $\alpha 2$ monomer is 215 amino acids and an MCM1 monomer is 285 amino acids, based on nucleotide sequence information for these genes^{5,26}. Adapted from refs 13 and 18 according to information from refs 17 and 114.

kinase^{46,47} and thereby cause arrest in G1 at 'start'⁴⁸, as occurs for mutants defective in the *CDC28* gene. Yeast cells thus exhibit a choice of either proliferation or differentiation depending on the presence of the mating factors. Cells can be induced by mating factors at stages other than G1 (ref. 41). Thus the link between cell-cycle arrest and differentiation may be due simply to the induction by the mating factors of a protein required to arrest the cell cycle (a candidate is the product of the *FAR1* gene; F. Chang and I. H., unpublished data).

Induction of gene expression by mating factors provides an opportunity to trace how a signal passes from the cell surface to the nucleus, where it stimulates gene expression. Although there are gaps in our understanding of the pathway, many of the genes and proteins involved have been identified (reviewed in refs 2, 49), and the outlines of a pathway can be seen (Fig. 3). The mating-factor receptors themselves, products of the *STE2* and *STE3* genes, are members of a family of receptors that includes rhodopsin and the beta-adrenergic receptor, all of which communicate with a tripartite G protein with alpha, beta

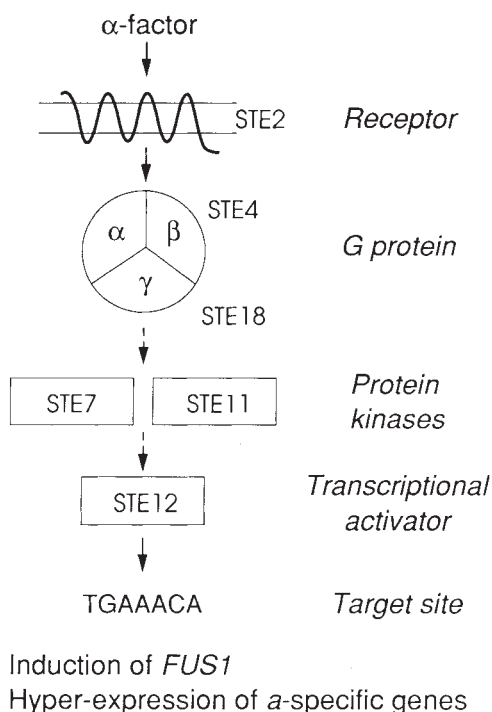


FIG. 3 Induction of differentiation by mating factors: the pathway of signal transduction from membrane to nucleus.

The diagram shows the pathway of signal transduction for *a* cells stimulated by α -factor. As described in the text, an activated receptor (an integral membrane protein located in the plasma membrane) causes the subunits of the G protein to dissociate. This ultimately leads to activation of STE12 protein, which (either by itself or with help from the MCM1 protein; see text) binds to the 'induction box' sequence in the upstream regulatory region of various genes, such as *FUS1* and the members of the *a*-specific gene set, to trigger transcription of these genes. Arrest of the cell cycle may result from transcriptional activation of another of these genes by STE12 (F. Chang and I. H., unpublished data; ref. 54). The manner in which STE12 becomes activated is not known, but it is speculated that the dissociated G protein in some way (indicated by a dotted arrow) activates the STE7 and STE11 proteins (which are believed to be protein kinases), which ultimately lead to activation of the STE12 protein. The alpha, beta and gamma subunits are encoded by genes *GPA1/SCG1* (refs 50, 110, 111) *STE4*, and *STE18* (ref. 108), respectively. Inactivation of any of the *STE* genes shown in the figure disrupts the response pathway—mutants neither arrest nor exhibit gene induction. Inactivation of the *SCG1/GPA1* gene leads to constitutive expression of the pathway, that is, gene induction and arrest even in the absence of α -factor. The pathway for signal transduction for α cells is the same as shown, except that the receptor is coded by the *STE3* gene; all of the intracellular machinery is identical in *a* and α cells.

and gamma subunits. Binding of the peptide ligands, *a*-factor or α -factor, to their receptors apparently causes the beta and gamma subunits of the G protein to dissociate from the alpha subunit and then trigger subsequent events through an unknown intracellular signal⁵⁰. Induction of gene expression by the mating factors can occur in the presence of cycloheximide, indicating that synthesis of new proteins is not required for induction^{40,41,51}. Protein kinases are likely to be involved in the pathway of signal transduction: the *STE7* and *STE11* gene products, which are required for response, show sequence similarity to protein kinases (ref. 52; B. Errede, personal communication) and act downstream of the G protein, although their precise roles and their substrates are not known. The trail becomes indistinct at this point but picks up later, in the nucleus.

A DNA sequence that confers inducibility by the mating factors and a protein that apparently binds to this site have been identified. A short sequence (TGAAACA—the 'induction box' or PRE, 'pheromone response element') is necessary for inducible response (ref. 25; see also refs 39, 51). These sites are present in different numbers of copies in the upstream regions of inducible genes. For *a*- and α -specific genes, these sites are located in different positions with respect to other regulatory sites—notably binding sites for MCM1 protein. The STE12 protein has recently been shown to bind to this sequence⁵³ and presumably is ultimately responsible for induction. An appealing hypothesis^{53,54} is that activity of the STE12 protein is governed by phosphorylation under control of the upstream protein kinases (Fig. 3). Other yeast activator proteins (heat-shock factor⁵⁵ and GAL4⁵⁶) are known to be phosphorylated under different conditions.

STE12 plays a part not only in induced expression but also in setting the basal level of expression of the *a*- and α -specific genes^{57,58}. Mutants defective in *STE12* exhibit modest but significant reductions (3- to 10-fold) in expression of *a*- and α -specific genes for strains grown in pure culture. Thus STE12 is needed for full basal expression but is not an absolute requirement. (The *STE7* and *STE11* genes are also required for basal expression^{40,42,58}, presumably by contributing to the basal activity of STE12.) A recent finding helps put the role of STE12 in perspective. Errede and Ammerer⁵⁴ have found that STE12 and MCM1 proteins interact: binding of STE12 to the upstream region of an *a*-specific gene requires MCM1. (This is the rationale for including MCM1-STE12 as another regulatory activity in Table 2.) It is not known whether STE12 can activate transcription by itself or whether it always requires interaction with another protein such as MCM1 (ref. 54). In any event, there seem to be three contributors to the expression of *a*- and α -specific gene sets: the most important is MCM1 protein (which, for α -specific genes, also requires $\alpha 1$); it is probably required under all conditions. The second contributor is STE12 protein in its 'unstimulated' state; it may stabilize MCM1 or otherwise enhance transcription and thereby contribute to basal expression. The third contributor is STE12 protein in the 'stimulated' state that results from activation of the signalling pathway. It is possible that the induced level of expression of *a*-specific and α -specific genes results from enhancement of the basal mode of expression.

We can now see that cells specialized for mating achieve their final, fully differentiated state by a combination of two processes: intrinsic programming and response to the environment. The final differentiation of an *a* or α cell is thus triggered at the appropriate time—when a mating partner is in the vicinity. In the context of developmental biology, it might be appropriate to view cells that have been induced by mating factors as distinct cell types (H. R. Horvitz, personal communication). An additional cell type is generated by induction (nutritional starvation) of the *a*/ α cell. By this method of reckoning, there are six cell types of yeast (Table 3).

Triggering differentiation at the appropriate time is also important during development of multicellular organisms. The

TABLE 2 Distribution of polypeptide subunits and regulatory activities in three different yeast cell types

a Polypeptide subunits					
Cell type	a1	α 1	α 2	MCM1	STE12
a	+	—	—	+	+
α	—	+	+	+	+
a/ α	+	—	+	+	—

b Regulatory activities					
a	α		a/ α		
MCM1	MCM1		MCM1		
	MCM1- α 1				
	MCM1- α 2		MCM1 α 2		
MCM1-STE12	MCM1-STE12		a1- α 2		

The indicated gene products are present in different cell types as shown in a. They interact with each other to form the regulatory activities shown in b. It is likely that STE12 can also act independently of MCM1, as dimers of STE12 binding sites are sufficient to create a mating-factor-inducible upstream activation site (S. Fields, personal communication).

embryological concept of induction (see ref. 59) is increasingly becoming accessible to molecular study at the level of the single cell. Differentiation of photoreceptor cells in the *Drosophila* ommatidium is thought to be a series of such inductive events, the final one being induction of the R7 precursor cell by the adjacent R8 cell^{60,61}. Another example comes from the nematode, in which the anchor cell induces differentiation of nearby vulval precursor cells⁶².

The process by which yeast cells mate demonstrates the phenomena of refinement and reinforcement, in which interacting cells generate a commitment to each other. As we have noted, cells express a basal level of mating pheromone and pheromone receptor. Thus a 'naive' α cell triggers a nearby a cell to synthesize more α -factor receptor (ref. 42), thereby increasing its responsiveness to α -factor, and also to synthesize more a-factor⁶³. This a-factor then stimulates the α cell to synthesize more a-factor receptor⁴¹ and more α -factor and so on. Obviously this process of mutual reinforcement (positive feedback) must be limited in some way. What happens, of course, is that the end product of the process—the a/ α cell formed by mating—turns off the machinery for synthesis of the mating pheromones and receptors. Similar signalling events may occur during *Drosophila* development (reviewed in ref. 64). For example, at borders of stripe patterns seen in the early embryo, adjacent cells might mutually induce each other to produce a unique differentiated state.

Regulatory hierarchy

As described above, the products of the *MAT* locus are regulatory proteins that determine cell type. Similarly, the products of the homoeotic genes of *Drosophila* determine the particular cell types present in a body segment (ref. 65; reviewed in ref. 64). One of the important questions in unravelling the molecular basis of development is to understand what determines which regulatory proteins are present or active in a given cell. In yeast, the *MAT* gene products are part of a regulatory hierarchy (Fig. 4). The *MAT* locus is at the 'top', coding for proteins that trigger expression of the specialized gene sets; and it is in turn controlled by the product of the *HO* gene, which determines which set of regulatory proteins is expressed in a given cell. The *HO* gene is itself regulated in several different ways.

Regulation of *MAT* by *HO*. The product of the *HO* gene does not encode a direct transcriptional regulator but operates a 'cassette' mechanism determining which *MAT* allele shall occupy the mating-type locus^{66,67} (Fig. 5). Although they are haploid, a and α cells in fact contain the information for both

MAT alleles, with only one allele actually occupying the *MAT* locus in any one cell. Only the allele at the *MAT* locus is active: two additional *MAT* genes, one copy of *MATa* and one of *MAT α* , are silent. The *HO* gene encodes a site-specific endonuclease⁶⁸ that initiates a genetic rearrangement in which the active allele at the mating-type locus is replaced by a copy of the opposite mating-type allele stored at the silent loci. As a result, the information from the silent copy is activated, and the mating type of the cell is switched. Thus, although *HO* encodes an endonuclease, its position in the regulatory hierarchy is formally that of a regulator of the master regulatory locus.

Regulation of *HO*. The hierarchy continues. The *HO* gene itself is subject to three different kinds of control: it is expressed only in a and α cells¹¹ (cell-type control); it is expressed only in late G1 (ref. 69) (cell-cycle control); and it is expressed only in certain cells of a mitotic lineage, mothers but not daughters⁷⁰ (mother-daughter or asymmetric control). *HO* is expressed only when these three conditions are met. The latter two types of control are responsible for generating mitotic cell lineages in which cells change from α to a and the reverse according to specific rules⁷¹ (Fig. 6a). One of the most striking observations is that the population is heterogeneous with regard to ability to switch mating types: some cells (mother cells) are competent to switch, whereas other cells (daughter cells) are not. Yeast cell divisions therefore generate a population containing a stem cell lineage, in which the original cell type is preserved and novel cell types are generated. Further analogies are shown in Fig. 6b.

The products of thirteen different genes are responsible for carrying out these different types of regulation. They act ultimately in an upstream region of the *HO* gene comprising 1,500 base pairs (bp), which has been subdivided into two broad regions known as upstream regulatory sequences (URSs)⁷²: URS1 is responsible for mother-daughter control, and URS2 is responsible for cell-cycle control (Fig. 7). Regulation is brought about by a1- α 2 (refs 11, 16) and the products of six *SWI* ('switch')^{38,73} and five *SIN* ('SWI-independent') genes^{74,75}. The *SWI* gene products are all necessary for transcription of *HO*, and they are therefore formally positive regulators of *HO*. The *SIN* products are thought to be negative regulators of *HO* expression. Some of these may be antagonized by *SWI* products.

The mechanism responsible for cell-type negative regulation of *HO* is straightforward: the *HO* upstream regulatory region is sprinkled with recognition sites for a1- α 2: ten sites are present throughout URS1 and URS2 (refs 16, 72). Binding of a1- α 2 to these sites presumably prevents activation events described below from occurring.

The sequences responsible for cell-cycle control of the *HO* gene have been identified. The URS2 region contains ten copies of a 'cell-cycle box', the conserved motif of which is CAC-

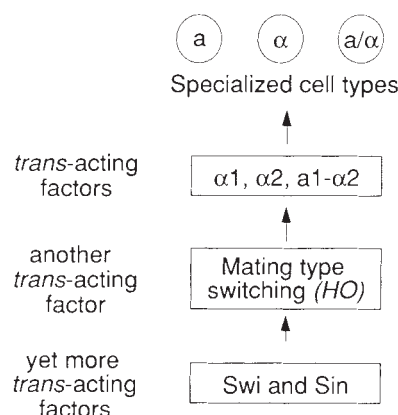


FIG. 4 Regulatory hierarchy for specialized cell types in yeast.

GAAAA (ref. 69). A DNA-binding activity that recognizes the cell-cycle box has also been identified (CCBF, for 'cell-cycle box factor')⁷⁶. SWI4 protein is present in the complex¹¹⁵; SWI6 is required for CCBF activity and may also be part of the complex. The basis for limiting *HO* expression to late G1, after the 'start' event of the cell cycle, is not yet known, but it may be that CCBF is phosphorylated by the CDC28 protein kinase, which regulates 'start'^{46,48}. Another possibility is that CDC28 phosphorylates another part of the transcription machinery, such as a subunit of RNA polymerase⁷⁷.

The restriction of *HO* expression to mother cells appears to be due to the behaviour of the regulatory proteins SWI5 and SIN3. The SWI5 product, which is required for transcription of *HO*³⁸, binds to a site in the URS1 region through its three zinc fingers^{78,79}. As SWI5 is not required in the absence of SIN3 (ref. 74), it is inferred that SWI5 activates transcription by antagonizing SIN3, which otherwise inhibits *HO* expression in some way. The important role of SWI5 and SIN3 in mother-daughter control is deduced from the observations that daughter cells can express *HO* if they are defective in *SIN3* (refs 74, 75) or if they overexpress the *SWI5* gene⁸⁰. It thus seems that mother and daughter cells differ from each other in their level of SIN3 activity, which is high in daughter cells and low in mother cells. The current working hypothesis is that this difference results from mother cells having a higher level of SWI5 than daughter cells, but the reason for this difference is not yet known. Preferential localization of SWI5 to mother cells or proteolytic degradation of SWI5 in daughter cells have been invoked as possible explanations.

Combinatorial control of *HO* expression. *HO* expression occurs in *a* or *α* cells only when two conditions are met: that the cells are mothers and that they are in the G1 phase of the cell cycle. Our working hypothesis (B. Andrews, W. Kruger, C. Peterson and I.H., manuscript in preparation) for this combinatorial control of *HO* involves sequential activation of the two upstream regulatory domains by two independent events, one occurring only in mother cells, the other only in late G1 (Fig. 7). In this scheme, SWI5 (through its action in URS1) initiates a sequence of events that culminates in the binding of CCBF to the cell-cycle box sequences in URS2. First, SWI5 'sweeps the deck', clearing the URS1 region of inhibitory factors such as SIN3. This allows another protein, which we call SWI1, 2, 3 (because it requires the *SWI1*, *SWI2* and *SWI3* genes) to act in URS1. Because SIN1 protein prevents the activation of transcription from the cell-cycle boxes located in URS2 (B. Andrews and W. Kruger, unpublished data), we have proposed that SWI1, 2, 3 activates

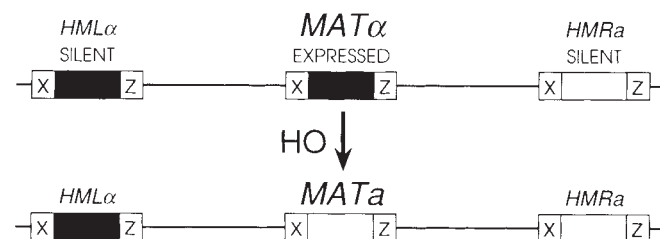


FIG. 5 Changing alleles of the mating-type locus by genetic rearrangement. The top line shows the arrangement of cassettes on chromosome III in an α cell. The cassette at *MAT* is expressed; those located at *HML* and *HMR* are repressed by Sir (see ref. 112). Switching to *a* occurs by removing the α cassette from *MAT* and replacing it by information from the silent version of *MATa*, which is located at *HMRa* (refs 66, 67). This process is initiated by the product of the *HO* gene, which is an endonuclease that produces a double-strand break at *MAT*^{68,113}. Subsequent repair of this break using the homologous sequences located at X and Z leads to a duplicative transposition of information from *HML* or *HMR* to *MAT*. The central regions of the cassettes (shown as solid or open rectangles) are nonhomologous segments described in the legend to Fig.1; the X and Z regions are ~700 and ~200 bp respectively. More details on the structure of the cassettes are given in ref. 5.

TABLE 3 Six cell types of yeast

Cell type	Properties
α cell	has the potential to mate
α cell induced by <i>a</i> -factor	has the ability to mate
<i>a</i> cell	has the potential to mate
<i>a</i> cell induced by α -factor	has the ability to mate
<i>a</i> / α cell	has the potential to sporulate
<i>a</i> / α cell induced by nutritional signal (starvation)	sporulates

transcription by antagonizing SIN1. Thus, in the next step in the sequence, SWI1, 2, 3 overcomes repression by SIN1. Then, CCBF is able to rush onto the scene, bind to the cell-cycle boxes and trigger transcription of *HO*. Given such an exhausting and complex scenario, it is no wonder that this process can occur only once each cell division and then only in mother cells. The particular form of complex regulation of the *HO* gene can thus be explained simply by control of the access of an activator protein (CCBF in this case) to its DNA binding site.

Although the molecular analysis of the *HO* upstream region is far from complete, a few additional points are worth making. First of all, the ability of the cell-cycle box element to function as a transcriptional activation site is strongly dependent on its location. When removed from URS2 and assayed in an artificial construct, it functions as an activating sequence⁷³. But in its normal context as part of the URS2 region, it functions as an activating sequence only if the URS1 region is present: if URS1 is deleted, activation from the cell-cycle box is eliminated⁷². This difference results from inhibition of the cell-cycle box in URS2 by SIN1, which, as we have seen, is itself regulated by events in URS1. I expect that there will be many such examples of regulatory elements from eukaryotic genes that exhibit this position-dependent behaviour. The second point that I would like to make is a speculation on how the complex *HO* regulatory region may have evolved. The present-day upstream region may have evolved by the juxtaposition of a URS1-like region to a DNA segment containing multiple cell-cycle boxes. This would yield a regulatory region that has tandem transcriptional activating segments, analogous to the regulatory region of the yeast *CYC1* gene⁸¹. In the case of *HO*, the two segments of the upstream region have become functionally linked, with the SIN1 protein acting as the coupling device. It operates in both regions, limiting access to the cell-cycle boxes in URS2, but itself being controlled by events that take place in URS1.

Several of the proteins that are involved in regulating *HO* are related to proteins found in other eukaryotes. Thus, the CDC28 protein kinase is related to the cdc2 protein kinase of fission yeast⁸², which is itself similar to a component of the general inducer of M phase, maturation promoting factor⁸³. Breeden and Nasmyth⁸⁴ identified a widely distributed motif coding for 33 amino-acid residues in *SWI6* that is present in the *cdc10* gene of fission yeast, as well as in the *Drosophila Notch* and nematode *lin-12* genes. The *SWI6*-cdc10 motif, whose function is unknown, has now been found in the *SWI4* gene¹¹⁵ and in the nematode *glp-1* gene⁸⁵.

Concluding comments

The role of negative regulation in cell-type-specific gene expression. Cell-type-specific expression of yeast genes involves an interplay of positive and negative regulatory factors. The MCM1 protein is likely to be an important transcription factor for yeast, with many sites throughout the genome. As we have seen, it is responsible for transcription of two specialized gene sets, the α - and *a*-specific genes, and it may also be necessary for transcription of essential genes such as *CDC28* (refs 23, 26). The positive factor that distinguishes α cells from *a* cells is the $\alpha 1$ protein, which assists MCM1 in activating transcription of

the α -specific gene set. The negative factor that distinguishes α and a cells is $\alpha 2$. MCM1 protein is fully capable of binding to the upstream regions of a -specific genes, even in α cells. But binding of MCM1 does not lead to transcription of these genes because $\alpha 2$ flanks it and thereby prevents MCM1 from functioning. Repression of transcription by $\alpha 2$ thus goes hand-in-hand with transcriptional activation by $\alpha 1$ in programming cell-type-specific gene expression.

Yeast cell specialization involves a second repressor activity, $a1-\alpha 2$. Although this negative regulator also contains the $\alpha 2$ polypeptide, it binds to a different site and almost certainly represses by a mechanism different from $\alpha 2$ (preventing proteins from binding rather than inhibiting their ability to activate transcription). Negative regulators such as $\alpha 2$ and $a1-\alpha 2$ provide an explicit demonstration of why the presence of a transcriptional activator protein may not be sufficient to activate gene expression. We think that there will be numerous other examples of negative factors acting at the level of DNA. The similarity between MCM1 and SRF makes it likely that analogues of $\alpha 2$ (and $\alpha 1$) will also be found in mammalian cells. Genetic evidence for negative regulators in *Drosophila* has recently been directly substantiated by the demonstration that the *eve* protein can repress transcription *in vitro*⁸⁶.

Cell-type transcription factors, master regulators of cell type, and hierarchies. The proteins that are ultimately responsible for the differences between a and α cells are encoded by the mating-type locus, which is thus the master regulatory locus that determines yeast cell type⁸⁷. Yeast has been a particularly favourable organism for identifying such a determinant: because yeast cells naturally differ at the mating-type locus⁸⁸, it was apparent that the alleles of this locus are the determinants for cell type^{4,89}. Many other organisms, in particular, fungi and algae, have natural alleles governing their life cycle which may also identify master regulatory loci for these organisms (reviewed in ref. 1). The route to identifying transcription factors responsible for cell specialization in multicellular organisms—potential master regulatory loci—is coming from several different avenues: from isolation of mutants, from identification of DNA-binding proteins, and from use of DNA transfection (reviewed in ref. 90). The *glass* gene, identified genetically, is a putative cell-type transcription factor necessary for development of the *Drosophila* photoreceptor⁹¹. The Pit-1 protein, identified by its binding to the upstream regions of mammalian prolactin and growth-hormone genes, may be responsible for pituitary-specific expression of these genes^{34,92,93}. A group of genes (the products of which are MyoD1, myogenin and Myf-5), cloned by transfection and subsequent cross-hybridization⁹⁴⁻⁹⁷, seem to be regulatory proteins that govern myogenesis in mammalian cells.

It may be instructive to view the transfection technique from the perspective of yeast cell specialization. The key feature of the transfection strategy is that it identifies a DNA segment (genomic or complementary DNA) that stimulates a recipient cell to undergo an identifiable change, such as production of proteins characteristic of a differentiated cell. The factor responsible for this behaviour must be dominant in its action, and it ultimately must activate transcription in the recipient cell. The key point is that this added factor is sufficient to trigger cell differentiation. The transfectant DNA might code for a transcriptional activator protein that is absent from the recipient cell. This would be analogous to adding $\alpha 1$ protein to a cells to activate transcription of the α -specific genes in these cells. Another possibility is that the transfectant DNA codes for a transcriptional repressor protein, which is lacking in the recipient cell, that blocks synthesis of a negative factor. This would be analogous to adding the $\alpha 2$ protein to an a cell, to generate $a1-\alpha 2$, which would repress synthesis of *RME1* and thereby allow expression of the sporulation-specific gene set (see Fig. 1).

The success of the transfection technique is critically dependent on finding a suitable recipient cell line⁹⁰. Indeed, the

MyoD1 protein can activate muscle-specific gene expression in a wide variety of cell types but not in all cell types⁹⁸. We can see precisely this behaviour if we consider the consequences of transferring the $\alpha 1$ gene into two different recipients⁹⁹. Synthesis of $\alpha 1$ in a cells leads to expression of the α -specific genes: the only thing lacking for expression of these genes is $\alpha 1$. By contrast, synthesis of $\alpha 1$ in a/α cells does not lead to expression of α -specific genes: the presence of $\alpha 1$ and MCM1 is not sufficient to activate transcription. This failure is a consequence of two kinds of repression exerted by $a1-\alpha 2$. First, $a1-\alpha 2$ represses synthesis of STE12 (ref. 100) and STE5 (J. Thorner, personal communication), both of which are necessary for maximal transcription of the α -specific genes^{57,58}. Second, at least some of the upstream regions of α -specific genes contain sites for $a1-\alpha 2$ (ref. 101). We thus see that there are many reasons why a/α cells do not transcribe the α -specific genes: they lack at least three proteins necessary for transcription of these genes, and they may also have a repressor bound to their upstream regions.

In the simplest view of things, MyoD1 is analogous to $\alpha 1$: its presence is sufficient to activate transcription, and it stands at the top of the hierarchy for cell specialization, where, like $\alpha 1$, it directly stimulates transcription of the members of the specialized gene set. Of course, less direct roles are also possible: MyoD1 might activate synthesis of other proteins that directly promote transcription of this gene set. In any event, the concept⁹⁸

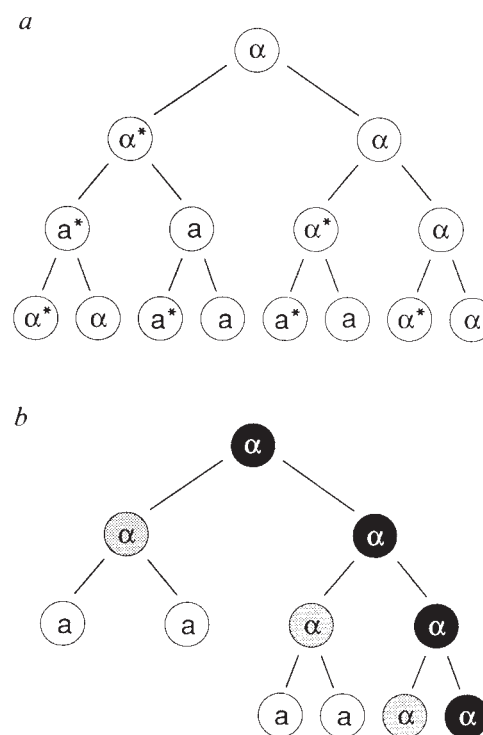


FIG. 6 Regulation of the cell-type regulatory genes generates a stem cell lineage. Panel *a* shows a mitotic cell lineage of yeast cells that carry the *HO* gene⁷¹. Mother cells are drawn to the left and daughters to the right at each cell division. Cells with an asterisk (cells that have undergone one cell-division cycle) are competent to produce cells with changed mating type in their next cell division⁷¹. This pattern results from two types of regulation of *HO*: it is transcribed only in mother cells and only in the G1 phase of the cell-division cycle (see text). Panel *b* accents analogies between the yeast cell lineage and a stem-cell/differentiated-cell lineage. The lineage of daughters (drawn in black) is a stem cell lineage; they give rise to cells like themselves at each cell division and also to cells (depicted as stippled) which are capable of giving rise to differentiated cells (drawn in white). In the yeast cell lineage, the 'white' cells can switch back to α ; however, if this cell lineage were conducted in the presence of α -factor, the 'differentiated' a cells would be terminal.

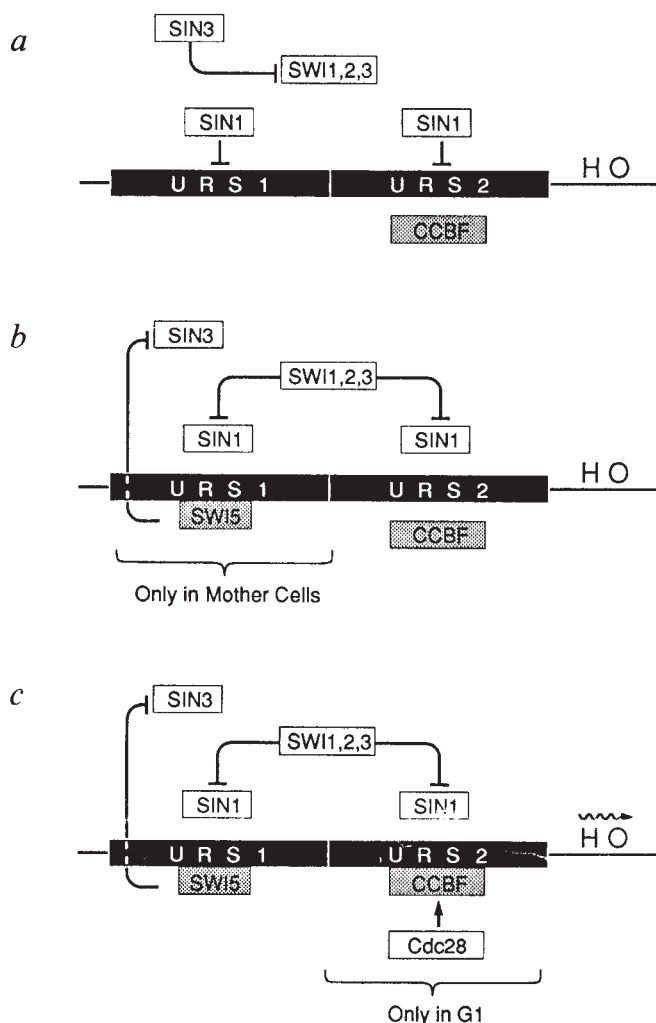


FIG. 7 Sequential activation scheme for expression of *HO*. The upstream regulatory region of *HO* contains two broad regions, URS1, which is responsible for mother-daughter regulation, and URS2, which is responsible for cell-cycle-dependent expression⁷³. URS2 contains ten short sequences (cell-cycle boxes; not shown) that confer cell-cycle-dependent expression and that are binding sites for CCBF (cell-cycle box factor). It is proposed that two independent events must occur to activate transcription of the *HO* gene—one event in URS1 (in mother cells) and another event in URS2 (in G1). Arrowheads indicate that a gene product stimulates a process or is necessary for a subsequent event; bars indicate that a gene product inhibits a process or another product. Inhibition of SWI1, 2, 3 by SIN3 and inhibition of SIN3 by SWI5 may occur by competition for binding sites in URS1. Only SWI5 and CCBF (drawn as shaded rectangles) are known to bind to DNA. Panel a shows the upstream regulatory region in a daughter cell, in which it is proposed that two negative factors (SIN1 and SIN3) keep the *HO* gene silent: SIN1 prevents access of CCBF to its target sites, the CCB elements located in URS2. SIN3 inhibits SWI1, 2, 3 until the appropriate conditions occur. Panels b and c show the series of events that is proposed to occur in mother cells according to the sequential activation scheme (B. Andrews, W. Kruger, C. Peterson and I. H., in preparation). First, SWI5 protein inhibits SIN3 (an event that occurs only in mother cells). As a result, the second step can occur: SWI1, 2, 3 inhibits SIN1 protein. Now that repression by SIN1 is lifted, the third and final event can take place (c): binding of CCBF to its target sites, the cell-cycle boxes. Activation of transcription by CCBF occurs only in the G1 phase of the cell cycle and requires the CDC28 protein kinase, which may stimulate activity of CCBF (as drawn) or phosphorylate other components of the transcription machinery. A major motivation for the scheme depicted here is that mutations that inactivate the *SIN1* and *SIN2* genes bypass the need for *SWI1*, *SWI2*, *SWI3*, and *SWI5* genes for transcription of *HO*, whereas *SWI4* (and presumably *SWI6*) are still required⁷⁴. The activity described as SIN1 may require also the *SIN2* gene; the activity described as SIN3 may require also the *SIN4* gene⁷⁴.

that MyoD1 represents a 'nodal point'—a point of potential regulation—in the pathway of muscle cell differentiation is a valuable one. Although it is clear that MyoD1 and relatives are sufficient to induce myogenesis, it is not known whether they are necessary and if they play this role in the whole organism. This will require inactivating the *MyoD1* gene, for example, in transgenic mice by gene replacement or by using dominant negative versions of MyoD1 (ref. 102). We may pursue the analogy between MyoD1 and $\alpha 1$ further and ask whether there is a mammalian analogue of MCM1: that is, whether MyoD1 itself activates transcription or whether it helps another protein to do so.

The regulatory hierarchy that is responsible for yeast cell type is crowned by sequence-specific DNA-binding proteins. Although DNA-protein recognition is one mechanism for generating specificity at a molecular level, and is used extensively for programming early *Drosophila* development (see, for example, ref. 103; reviewed in ref. 64), it is by no means the only mechanism. Indeed, the yeast hierarchy demonstrates the use of genetic rearrangement to control the choice of regulatory proteins. And the genetic hierarchy used for sex determination by *Drosophila* makes extensive use of alternative splicing to generate male- and female-specific pathways of development¹⁰⁴. The steps in a regulatory hierarchy may use a wide variety of molecular mechanisms, including some not described here (such as proteolysis; see ref. 87).

Devices for monitoring progress and state. The process of development in multicellular organisms must include ways of monitoring the status of a cell and whether specific events have been executed. One of the important strategies for monitoring is the

use of negative feedback control, whereby end products of a pathway signal their presence by inhibiting their further synthesis. The heteromeric repressor species, $\alpha 1$ - $\alpha 2$, is used as such a monitoring device in several different respects. First of all, $\alpha 1$ - $\alpha 2$ provides a molecular signal that successful mating to produce a diploid cell has been accomplished. Diploidy *per se* is not monitored: diploid α/α or α/α cells (produced by mitotic recombination from α/α cells) behave as their respective haploids in that they produce mating factors and so on. It is $\alpha 1$ - $\alpha 2$, composed of products from both mating partners, that gives the α/α cell its identity. Repression of *HO* by $\alpha 1$ - $\alpha 2$ is another good example of negative feedback regulation, in this case, a process that involves genetic rearrangement (see refs. 1, 11). As noted above, positive feedback also plays an important role in the mating process, whereby partially differentiated cells induce each other's final differentiation. It is intriguing that the upstream regulatory region of the *STE12* gene contains several sites for induction^{53,54}, which might serve either for positive or negative feedback.

One of the strategies that is used repeatedly for monitoring status and progress in yeast is that of combinatorial control. There are different types and levels of such control, and we have already seen one: the formation of a novel regulatory species, $\alpha 1$ - $\alpha 2$, by combining polypeptides. Combinatorial control is also responsible for restricting mating-type switching, not only to certain cell types, but also to when it occurs in the cell cycle and in which cells it occurs. Three independent conditions must be satisfied before the *HO* gene is expressed. Looking at the *HO* upstream regulatory region (at present primarily using our genetic eyeglasses), we can see the molecular basis for this

combinatorial control. In this case, we think that events in one segment of DNA are coupled to events in another DNA segment in a dependent manner.

Violins and orchestras. Because yeast is a single-cell organism, one might at first have thought that its molecular and cellular sophistication would be modest. However, yeast cells do a lot more than simply double every 100 minutes: they can also mate and sporulate. It is the cells that are specialized for these life-cycle transitions which exhibit such interesting properties and

which potentially shed light on differentiation in multicellular eukaryotes. Yeast presents an integrated picture of how regulatory proteins and intercellular signalling fit together to determine cell type. In trying to unravel the logical and molecular basis of development in multicellular organisms, it may be helpful to consider what a single cell can do. □

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Observational determination of the greenhouse effect

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Satellite measurements are used to quantify the atmospheric greenhouse effect, defined here as the infrared radiation energy trapped by atmospheric gases and clouds. The greenhouse effect is found to increase significantly with sea surface temperature. The rate of increase gives compelling evidence for the positive feedback between surface temperature, water vapour and the greenhouse effect; the magnitude of the feedback is consistent with that predicted by climate models. This study demonstrates an effective method for directly monitoring, from space, future changes in the greenhouse effect.

146 W m^{-2} whereas clouds increase G by 33 W m^{-2} . G increases significantly with surface temperature (T_s), even when it is normalized by the surface emission. The rate of increase of G , $3.3 \text{ W m}^{-2} \text{ K}^{-1}$, is consistent with the magnitude of the H_2O greenhouse feedback inferred from climate models and line-by-line radiative-transfer calculations. Furthermore, the observed variation of total atmospheric water content (W) with T_s indicates that the normalized greenhouse effect (g) is strongly correlated with W . The observations are compelling evidence for the H_2O feedback. The data also reveal a rapid rise in G at the higher temperatures ($T_s > 298 \text{ K}$), the cause(s) of which are not apparent.

IN its normal state, the Earth-atmosphere system absorbs solar radiation and maintains global energy balance by re-radiating this energy to space as infrared or longwave radiation. The intervening atmosphere absorbs and emits the longwave radiation, but as the atmosphere is colder than the surface, it absorbs more energy than it emits upward to space. The energy that escapes to space is significantly smaller than that emitted by the surface. The difference, the energy trapped in the atmosphere, is popularly referred to as the greenhouse effect, G . Some very interesting questions about the greenhouse effect involve feedbacks between the various elements of the climate system¹. These feedbacks tend to enhance or diminish small external perturbations in the radiation balance. One of the most important is the water-vapour feedback². Consider an initial increase in the global temperature because of the direct infrared trapping resulting from, say, an increase in carbon dioxide. As the surface and the atmosphere warm, the saturation vapour pressure increases exponentially with temperature, and more H_2O can evaporate into the atmosphere. Because water vapour is a strong greenhouse gas, the extra water vapour traps more longwave radiation and drives the temperature even higher. This feedback can amplify the original perturbation by as much as a factor of two^{1,2}.

The greenhouse effect and its feedback on the climate have been explored by hundreds of theoretical and model studies. Satellite radiation-budget measurements have also been used to examine the radiative feedbacks in the climate system³. Yet to our knowledge the radiative trapping by the atmosphere has not been examined in depth with observed data (with the exception of unpublished work by R. D. Cess). Here we address three critical issues: (1) the magnitude of G in the present atmosphere; (2) the quantitative nature of the feedback between surface temperature and G ; (3) the change in G with time. We provide observational estimates for the former two and discuss how our findings bear on the third issue. A possible change in G is of urgent concern because of the rate at which human activities have been adding greenhouse gases to the atmosphere.

We obtain G by subtracting longwave radiation escaping to space from estimates of the radiation emitted by the ocean surface. The radiation escaping to space has been measured since 1985 by the spaceborne Earth Radiation Budget Experiment (ERBE). Under cloudless skies, the atmosphere traps

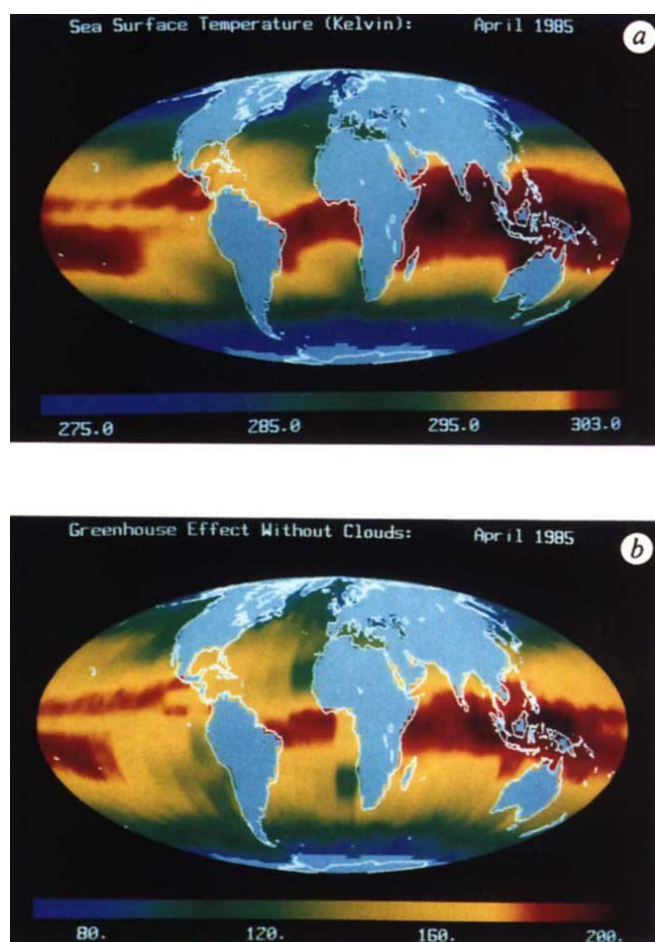


FIG. 1 *a*, Monthly-mean map of observed sea surface temperature (K) for April 1985. *b*, Same as in *a* but for the greenhouse effect (G) of atmosphere under clear skies. Values are in W m^{-2} . The major inference from these figures is that the greenhouse effect of the atmosphere is strongly correlated with surface temperature. This correlation can explain the Equator to pole decrease in G as well as the east to west variations. For example, G is maximum over the relatively warm western equatorial Pacific Ocean (Indonesian region) and is very low over the cold oceanic upwelling regions in the central and eastern equatorial Pacific and off the west coasts of Africa and South America.

Determination of the greenhouse effect

If E is the longwave flux emitted by the surface at a certain location, and F is the flux leaving the top of the atmosphere (TOA) directly above that location, then the greenhouse effect G for that location can be defined as $G = E - F$.

The TOA fluxes F have been measured with good accuracy by scanning radiometers on board the spaceborne ERBE⁴. ERBE consists of three satellites carrying identical scanners that detect both longwave (infrared) and shortwave (solar) radiation. The ERBE scanners, when viewing directly down, can resolve an area of $\sim 35 \times 35$ -km. The scanners have in-flight calibration which assures an accuracy of 1% for the satellite-altitude readings. Those readings are converted to TOA fluxes on a global grid of $2.5^\circ \times 2.5^\circ$ (latitude $\times$ longitude). So far, we have processed data for the months of April, July and October 1985 and January 1986. A unique feature of ERBE is that it separates the fluxes for clear skies (cloudless conditions) from fluxes for cloudy skies (mixture of clear and overcast areas)⁵. Hence we can obtain the greenhouse effect of the atmosphere and that of clouds separately.

We estimate the emission from the surface using the Stefan-Boltzmann black-body law ($E = \sigma T^4$) and the observed surface temperature. Because, in the infrared, the ocean surface approximates a blackbody to within 1% (ref. 6), and because the diurnal surface temperature variation is small in the ocean's mixed layer (less than a few degrees K), the uncertainties in the calculated values of E are minimum over oceans. We restrict this study to the open oceans; that is, we ignore continents and ocean regions covered with sea ice.

We obtained surface temperatures (SSTs) from the National Meteorological Center daily data sets archived at the National Center for Atmospheric Research (NCAR). The SST values are derived by blending *in situ* (ship and buoy) data with satellite temperature retrievals⁷.

The SSTs used during the period of this study have an estimated error of about ± 1.0 K (ref. 7). The error in the surface emitted flux is simply $\Delta E = 4\sigma T^3 \Delta T$, or about $\pm 5 \text{ W m}^{-2}$. ERBE TOA longwave fluxes have an estimated error of about $\pm 5 \text{ W m}^{-2}$ for monthly averaged values^{4,5}. Because the errors in E and F are uncorrelated, the error in the monthly averaged greenhouse effect for a single location is $\pm 7 \text{ W m}^{-2}$. When these values are averaged around a latitude belt, the random error will diminish significantly. Clear-sky fluxes, however, can have systematic errors that are due to cloud contamination. The magnitude of this error was examined⁵ by sorting out homogeneous cloudless regions, that is cloudless regions that are surrounded by other cloudless regions and for which the spatial standard deviation of the longwave flux is $< 1\%$ of the clear-sky flux. This stringent homogeneity criterion gave fluxes that were smaller than the operational values used here by only 4 W m^{-2} , indicating minimal cloud contamination⁵. In addition, the clear-sky fluxes when compared with state-of-the-art radiation models agreed within 1% (ref. 5). Overall, we expect the error (systematic plus random) in the monthly and regional mean values of G to be 5 to 10 W m^{-2} .

Global greenhouse effect of atmosphere and clouds

Estimates of G over the open oceans for a cloudless atmosphere (clear sky) reveal significant regional variations (Fig. 1b). Globally, for April 1985, the surface emission is 421 W m^{-2} whereas the TOA emission is 243 W m^{-2} , resulting in a total trapping of 178 W m^{-2} . Of this, the atmospheric gases (H_2O , CO_2 and O_3 among others) trap 146 W m^{-2} , whereas clouds trap the remaining 33 W m^{-2} . The greenhouse effect for each of the months studied is given in Table 1.

As the months are taken from the four seasons, we will assume the four month mean to be representative of the annual average. Then the annual average value of G (179 W m^{-2}) is comparable to the global mean solar absorption, $\sim 237 \text{ W m}^{-2}$. The global

mean latent heat released in the atmosphere is $\sim 100 \text{ W m}^{-2}$. The greenhouse effect of a doubling of CO_2 is 4 W m^{-2} and that of human activities during the past century⁸ is $\sim 2 \text{ W m}^{-2}$. The greenhouse effect of the atmosphere, next to solar absorption, is the largest factor in maintaining the present climate.

The TOA flux is given by the radiation transfer equation

$$F = B(T_s) - \int_0^1 A(x) [dB/dx] dx \quad (1)$$

This equation is written in a normalized pressure coordinate, $x = p/p_s$, where p_s is the surface pressure. The blackbody emission B is simply $\sigma T^4 = \int B_\lambda(T) d\lambda$, where B_λ is the monochromatic Planck function evaluated at atmospheric temperature T . The effective absorptivity A is the integral of the monochromatic absorptivity A_λ weighted with dB_λ , and normalized by dB (ref. 2). Thus $A(x)$ is the absorptivity between the TOA ($x = 0.0$) and the pressure level x . T_s is the surface temperature. Because $G = E - F$ and $E = B(T_s)$, we obtain

$$G = 4\sigma \int_0^1 A(x) T^3 [dT/dx] dx \quad (2)$$

where we have let $(dB/dx) = 4\sigma T^3 (dT/dx)$. Most of the contribution to A comes from the troposphere (the bottom 10 km) which contains the bulk of the absorber mass. (Although CO_2 is uniformly mixed in the atmosphere, the major absorbers—water vapour and clouds—are almost entirely restricted to the troposphere.) In the troposphere temperature generally decreases with height. Hence $(dT/dx) > 0.0$ and G is positive. This reveals that for G to be positive, the temperature must decrease with altitude in the region of the absorbing gas.

Evidence of water vapour feedback

The clear-sky G (Fig. 1b) generally decreases from Equator to pole with some exceptions. The trapping that is due to clouds (not shown here) does not vary systematically with latitude, but peaks in the convectively disturbed regions of low latitudes and in the vicinity of the mid-latitude jet-stream regions. We find that the clear-sky G has a strong positive correlation with sea surface temperature (compare Fig. 1a with b). Here we show that this correlation gives direct evidence of the H_2O feedback.

We do this in three steps. First, we show the correlation of G with T_s implies that the longwave absorptivity of the atmosphere increases with surface temperature. Second, the variation of water vapour in the atmosphere is determined largely by the increase in the saturation vapour pressure with temperature, as shown by other observational^{2,9} and modelling¹⁰ studies. Third, if the observed variation of water vapour causes the sort of G - T_s coupling revealed by the data, the atmosphere absorptivity must scale logarithmically with the water content. This is not only indicated by the observations but is also consistent with theories of radiative transfer.

Step 1. Because the surface emission increases as the fourth power of T_s , the trapping should also increase with T_s . To remove the T_s^4 dependence, we define a normalized trapping $g = G/\sigma T_s^4$. Both the clear-sky g (Fig. 2) and the cloudy g (not shown) increase nonlinearly at warm temperatures ($T_s > 298 \text{ K}$), but otherwise g increases smoothly with T_s . The grid-point

TABLE 1 Monthly- and global-mean greenhouse effect (W m^{-2})

Month	Clear sky	Clouds	Cloudy sky*
April 1985	146.3	32.1	178.4
July 1985	147.7	31.3	179.0
October 1985	146.9	32.9	179.8
January 1986	143.7	34.1	177.8
Four-month mean	146	33	179

* Cloudy sky is the sum of clear sky and clouds.

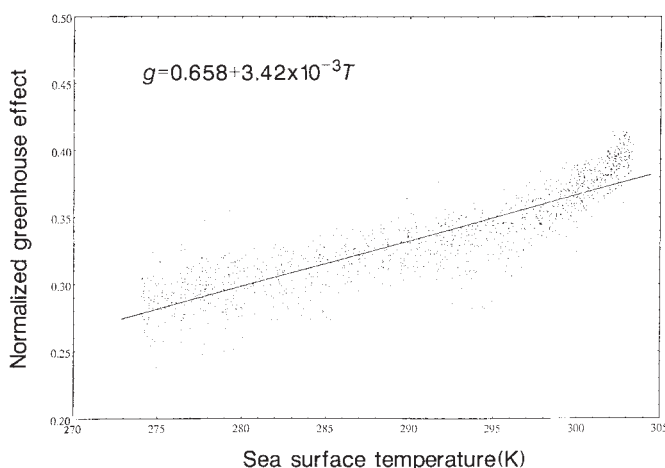


FIG. 2 Clear-sky greenhouse effect normalized by infrared emission (g) as a function of surface temperature, April 1985. Values are monthly averages for each $2.5^\circ \times 2.5^\circ$ region. The solid line indicates a least-squares fit through all the points. Fits done individually through Northern and Southern Hemisphere points do not deviate by more than 3%.

values of g for the individual months and the two hemispheres reveal similar dependencies on T_s . Because of the robustness of this feature, we conclude that g and the surface temperatures are strongly coupled.

The increase in g is primarily because of the increase in the absorptivity with T_s . In the troposphere the lapse rate $\Gamma = -dT/dz$ is approximately constant with pressure. We can then express the atmospheric temperature as $(T/T_s) = x^\alpha$, where $\alpha = \Gamma R/g_a$, where g_a is the gravitational acceleration. Inserting this in equation (2)

$$g = G/\sigma T_s^4 = 4\alpha \int_0^1 A(x)x^\beta dx \quad (3)$$

where $\beta = (4\alpha - 1)$. We first see that g does not explicitly depend on T_s . The g - T_s coupling must arise because either the lapse rate (and hence α) or A depend on T_s . The observed change in lapse rate¹¹ over the range of T_s considered here can affect g by at most 10% (based on flux sensitivities to lapse rates given in ref. 12). The only other variable is A . For a fixed amount of H_2O , A varies by only 5% (ref. 2) for the range of T_s considered here. The temperature dependence of A also cannot explain the rate of increase of g with T_s . Therefore, the amount of water vapour must increase with surface temperature.

Step 2. The increase in water vapour with T_s , which in turn causes an increase in A , is sufficient to explain the g - T_s coupling. The saturation vapour pressure e_s of H_2O is given by the Clausius-Clapeyron equation $C \exp\{-(LR^{-1})/T\}$. Here, C is a constant, L is the latent heat of vaporization and R is the gas constant for water vapour; LR^{-1} , at the global mean temperature, is $\sim 5,400$ K. Using the observed global mean lapse rate and relative humidity profile² we numerically calculate the total water content W in a vertical column in the troposphere for a range of values of T_s . W increases exponentially with a rate of increase $d(\ln W)/dT_s = 6.7 \times 10^{-2} \text{ K}^{-1}$. Compare this to the actual rate of increase ($5.5 \times 10^{-2} \text{ K}^{-1}$), obtained by satellite microwave observations¹³ of W (Fig. 3a). They agree to within 20%, hence W is largely determined by the surface temperature, in agreement with the findings of Prabhakara *et al.*⁹ and Stephens¹⁴. This coupling between W and T_s arises from the temperature dependence of the saturation vapour pressure.

Step 3. Because we are ignoring variations in the lapse rate or in spectroscopic parameters with temperature, we can write dg/dT_s as a product of a partial and a total derivative:

$$\frac{dg}{dT_s} = \frac{\partial g}{\partial \ln W} \cdot \frac{d \ln W}{dT_s} \quad (4)$$

The first term is the observed coupling of the normalized greenhouse effect with T_s . The last term represents the fundamental source of the H_2O feedback—the water content increases with T_s . The second term represents the ‘return loop’ in the feedback—the absorptivity increases with water content. We infer from equation (4) that if g varies linearly with T_s (which it does for $T_s < 298$ K in Fig. 2), and if $\ln W$ also varies linearly with T_s (as suggested from theory and from the satellite microwave data in Fig. 3a), then g should increase logarithmically with W . In fact, theoretical calculations² (see ref. 15) have shown that, for the range of W considered in Fig. 3, the absorptivity scales logarithmically with the absorber amount. A plot of g against $\ln W$ (Fig. 3b) shows this scaling. Our analysis is consistent, because dg/dT_s (from Fig. 2) is $3.4 \times 10^{-3} \text{ K}^{-1}$, on a global average, whereas the product of $\partial g/\partial(\ln W)$ (from Fig. 3b) and $d(\ln W)/dT_s$ (from Fig. 3a) is $3.2 \times 10^{-3} \text{ K}^{-1}$. This comparison may be used in an illustrative sense only, because the microwave soundings of W are only available for the period 1979–83, and the ERBE measurements are not available before 1985. However, a comparison of zonal averages (as shown in the plot of g against $\ln W$ in Fig. 3b) minimizes the variation across these two time periods.

The analysis strongly indicates the presence of the H_2O feedback. Three further points can be made in favour of this interpretation. First, the clear-sky greenhouse effect and its temperature

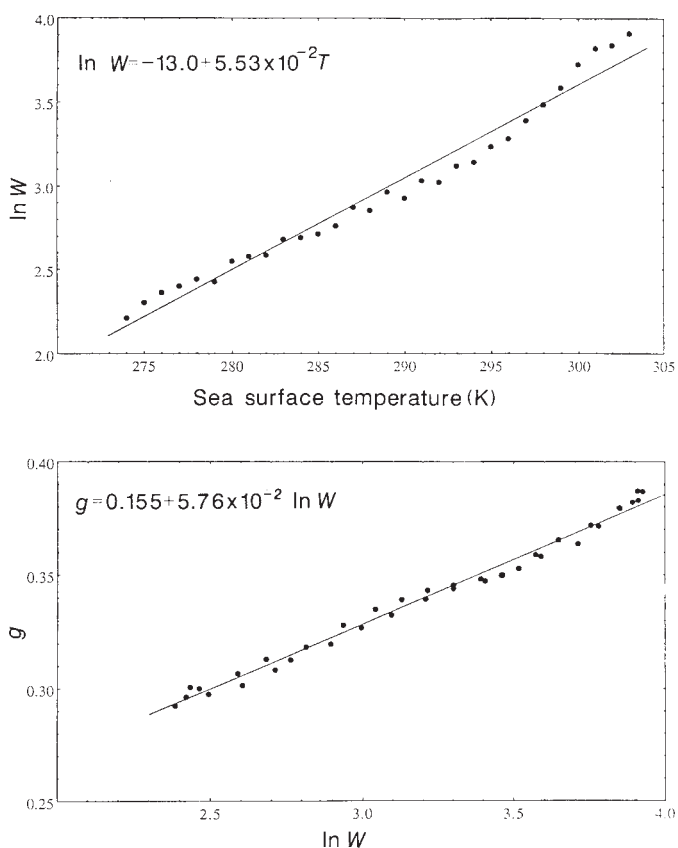


FIG. 3 a, Natural logarithm of the atmospheric water content (W , kg m^{-2}) as a function of surface temperature, April 1982, monthly averages for $3^\circ \times 5^\circ$ regions. The water content was derived from microwave satellite soundings¹³ available for 1979–1983. The error in W is $\sim 10\%$ (ref. 13). The strong positive correlation indicates that the behaviour of W follows simple thermodynamic laws; the slight upward and then downward deviations are consistent with the latitudinal variations in relative humidity² and lapse rate¹¹ which are governed by the dynamics of the atmosphere. b, Normalized clear-sky greenhouse effect (g) for April 1985 plotted against $\ln W$ for April 1982. ERBE data is not available before 1985 whereas W is not available after 1983. To minimize the effect of year to year variations in W , we have used zonally averaged values.

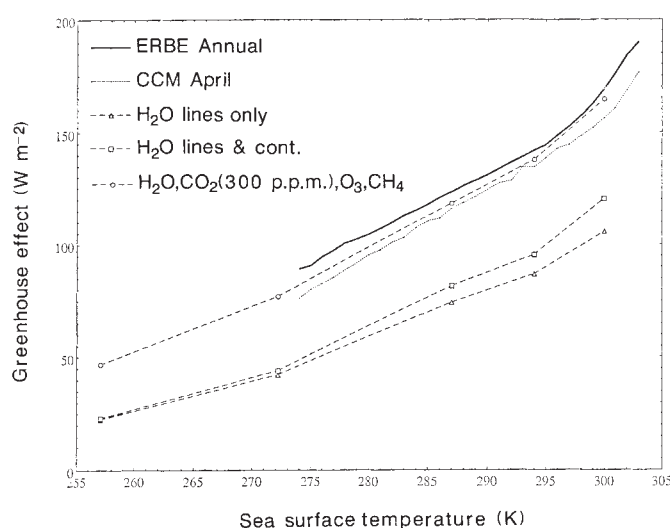


FIG. 4 Comparison of G and surface temperature, retrieved from three sources: bold line, ERBE annual values, obtained by averaging April, July and October 1985 and January 1986; thin line, three-dimensional climate-model simulations for a perpetual April (National Center for Atmospheric Research, Community Climate Model); dashed lines, line-by-line radiation-model calculations by Dr A. Arking (cited in ref. 16) for individual H_2O spectral lines, as well as the continuum absorption. The five points use climatological winter and summer atmospheric profiles for subarctic and mid-latitude conditions, and a tropical profile. A comparison of the calculated G with and without the continuum absorption (bottom two dashed lines) indicates a nonlinear contribution from the continuum. This may in part account for the nonlinear rise at the highest temperatures in the ERBE-derived G . When CO_2 , O_3 and CH_4 are added (the top dashed line) the line-by-line model results come close to ERBE values.

dependence is consistent with state-of-the-art radiative-transfer calculations¹⁶ (Fig. 4). These calculations use observed humidity and temperature profiles for each climate type (subpolar winter, midlatitude summer, for example). Hence, their agreement with the observed G indicates that the 'return loop' in the H_2O feedback, $\partial g / \partial (\ln W)$, behaves as expected from theory. Second, the sensitivity dG/dT_s of the NCAR Community Climate Model (CCM)¹⁷ agrees closely with the observed sensitivity (Fig. 4). The CCM is a three-dimensional general circulation model. The model ignores the radiative effects of trace gases such as CH_4 and N_2O (which contribute $\sim 5\text{--}7\text{ W m}^{-2}$ to the clear-sky G) and is also drier than the observed climate¹⁸. Hence it systematically underestimates G by $10\text{--}15\text{ W m}^{-2}$. It has, however, a latitudinal sensitivity dG/dT_s of $3.1\text{ W m}^{-2}\text{ K}^{-1}$, which compares well with the value of $3.3\text{ W m}^{-2}\text{ K}^{-1}$ obtained from ERBE, averaged annually and over the whole range of sea surface temperatures.

Finally, the general circulation model (GCM) designed by S. Manabe shows a climate sensitivity consistent with the analysis of ERBE flux observations. Wetherald and Manabe calculated¹⁹ that a 2% increase in the solar constant increased the GCMs

outgoing longwave flux by 6.3 W m^{-2} , and the surface temperature increased by 3.0 K. Thus the longwave trapping of the GCM has a sensitivity to the global mean temperature of $dG/dT_s = 4\sigma T_s^3 - dF/dT_s$, or $3.7\text{ W m}^{-2}\text{ K}^{-1}$. A 2% decrease in the solar constant results in a higher sensitivity, $4.0\text{ W m}^{-2}\text{ K}^{-1}$. The cloud cover is held constant in this model, hence the cloud-cover feedback is zero. The observed sensitivity is within 15% of the GCM values. This implies that the climatological sensitivity of this GCM is in good agreement with the latitudinal sensitivity of the present atmosphere. Such behaviour is necessary if the change in the equilibrium climate is determined largely by the H_2O greenhouse feedback.

The analysis up to this point is very encouraging—the independent climate data sets show an overall consistency that can be explained in terms of thermodynamics and radiation physics. This is perhaps one of the best indicators of the accuracy of satellite data. We find compelling evidence that the H_2O feedback is detectable from ERBE observations and that it can account for most (at least 80%) of the observed variation of G with surface temperature. Other atmospheric processes, such as the variation of relative humidity or lapse rate with latitude, are needed to explain the remaining variation.

Implications to the global warming problem

The coupling between surface temperature and the greenhouse effect will lead to a strong positive feedback on any perturbations to the present climate. One significant implication is that we might detect the greenhouse effect of human activities. The increase in trace gases, if it continues unabated, will directly increase G by about 0.5 W m^{-2} per decade. The cumulative increase in G for the next 50 years will then be $\sim 2.5\text{ W m}^{-2}$. In the same period, the globe is expected to warm by 2–4 K (ref. 8). Because of the coupling between T_s and g , the global G will actually increase by a further $6.5\text{--}13\text{ W m}^{-2}$, leading to a total increase of $9\text{--}15.5\text{ W m}^{-2}$. Such an increase should be clearly detectable if we continue ERBE-type observations into the next century. If the tropical regions exhibit the nonlinear increase revealed by the ERBE data (Fig. 2), the effect should be most prominent in the tropics.

Both the cloudy and clear-sky greenhouse effect increases rapidly at temperatures characteristic of the tropics. It is at present not known whether this nonlinear rise is simply a climatological feature of low latitudes, unrelated to temperature variations. For example, the relatively dry regions found in the subtropics ($290 < T_s < 298\text{ K}$) may slightly suppress the rate of increase of g with T_s and hence change the slope.

The nonlinearity might, on the other hand, be due to the nonlinear dependence of the H_2O continuum opacity¹⁶ or a temperature threshold for deep convection²⁰. Because deep convection is a source for the ice content of cirrus clouds, the greenhouse effect of cirrus clouds can increase with T_s when T_s exceeds a certain threshold value. If so, the magnitude of the positive feedback is so large that we should look for a compensating negative feedback elsewhere in the system to explain the relative stability of the tropical climate during the last great ice age. □

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Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel

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The complete amino-acid sequence of the cyclic GMP-gated channel from bovine retinal rod photoreceptors, deduced by cloning and sequencing its complementary DNA, shows that the protein contains several putative transmembrane segments, followed by a region that is similar to the cyclic GMP-binding domains of cyclic GMP-dependent protein kinase. Expression of the complementary DNA produces cyclic GMP-gated channel activity in *Xenopus* oocytes.

which suggests that this polypeptide alone is sufficient to form a functional cGMP-gated channel.

Cloning of cDNA

The initial approach to isolating cDNA for the cGMP-gated channel was to screen a cDNA library by hybridization with oligodeoxyribonucleotide probes synthesized on the basis of partial amino-acid sequence data. The cGMP-gated channel polypeptide of M_r 63K was solubilized from bovine rod outer segments and purified as described previously¹². After SDS-PAGE of the purified preparation, the 63K polypeptide was electroeluted from the gel and digested with trypsin. The resulting peptides were fractionated by reverse-phase HPLC (Fig. 1). Fifteen fractions (I–XV) were collected and analysed for amino-

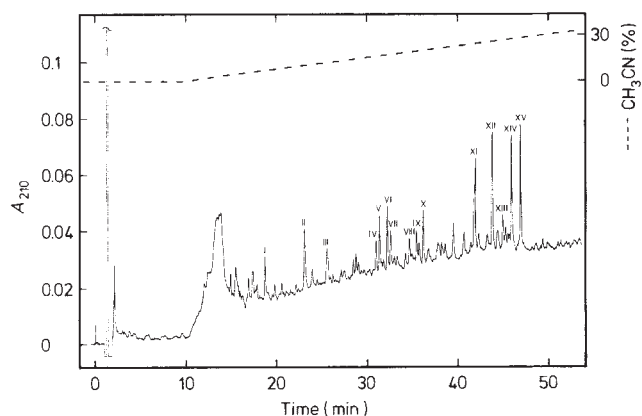


FIG. 1 Reverse-phase HPLC of tryptic peptides derived from the cGMP-gated channel. Solid line and left-hand axis, absorbance at 210 nm (A_{210}); broken line and right-hand axis, acetonitrile concentration. The amino-acid sequences determined for the fractions (I–XV) were identical to those of the following amino-acid residues deduced from the cDNA sequence (for amino-acid numbers, see Fig. 2): I, 639–641; II, 406–411; III, 591–596; IV, 454–460; V, 417–422; VI, 642–652; VII, 240–245; VIII, 513–518; IX, 668–677; X, 628–637; XI, 261–269; XII, 279–284; XIII, 435–443; XIV, 663–676; XV, 435–443. Identical amino-acid sequences were determined from fractions XIII and XV, and the sequence for fraction XIV overlapped that for fraction IX. METHODS. The 63K protein was purified as described previously¹². After SDS-PAGE of the purified preparation, a piece of the gel carrying the 63K polypeptide was excised and the protein was electroeluted and dialysed³⁸. The peptides resulting from tryptic digestion of the dialysed material (330 pmoles) were fractionated by reverse-phase HPLC and sequenced; the procedures used were essentially identical with those described previously³⁹.

VERTEBRATE photoreceptors respond to light by a transient hyperpolarization due to the closure of a cation channel in the plasma membrane. The channel is activated directly¹ by guanosine 3',5'-cyclic monophosphate (cGMP), the internal messenger of visual transduction (for reviews, see refs 2–4). The channel does not discriminate very well between alkali cations^{1,5–8} and is reversibly blocked by divalent cations^{9–11} and *l*-cis-diltiazem⁹. The cGMP-gated channel from bovine retina consists of a single type of polypeptide of relative molecular mass (M_r) 63,000 (63K)¹². The purified bovine cGMP-gated channel is functional when reconstituted into phospholipid vesicles¹³ or artificial planar bilayers¹⁴. Channel activation occurs by the cooperative binding of at least three^{10,11}, but probably more^{3,12,13}, cGMP molecules, which suggests¹² that the functional channel is composed either of several 63K polypeptides each harbouring one or two cGMP-binding sites, or of only a single polypeptide containing three or more cGMP-binding sites. It has also been proposed that the cGMP-gated channel is composed of polypeptides of M_r 39K¹⁵ or M_r 250K¹⁶ or of rhodopsin¹⁷ itself, which raises the possibility¹⁸ that the channel is composed of different polypeptides, that the 63K polypeptide is a dimer of the 39K polypeptide or that the 39K polypeptide results from proteolytic modification of the 63K polypeptide. Alternatively, these polypeptides may represent different cGMP-gated channels.

We have now cloned DNA that is complementary to bovine retinal messenger RNA coding for the 63K polypeptide and from the nucleotide sequence analysis of this cDNA we are able to predict the complete amino-acid sequence of the polypeptide. The cDNA has been functionally expressed in *Xenopus* oocytes,

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FIG. 4 Amino-acid sequence similarity between a region of the cGMP-gated channel and the cGMP-binding domains of cGMP-dependent protein kinase (cGK). Amino-acid residues 498–577 (in one-letter code) of the cGMP-gated channel are aligned with the sequences of the two cGMP-binding domains of bovine lung cGK²³ (referred to as cGK (1) and cGK (2)); numbers of the amino-acid residues at both ends are given. Identities between the cGMP-gated channel and cGK (1) or cGK (2) are indicated by solid boxes, and conservative substitutions⁴⁷ by dotted boxes. Between the cGMP-gated channel and cGK (1), 32% of the aligned positions are occupied by identical

residues, and 57% by identical or conserved residues; the probability that the sequence similarity occurs by chance²² is 1.2×10^{-11} . The corresponding degrees of similarity between the cGMP-gated channel and cGK (2) are 30% and 51% (probability, 5.2×10^{-11}). For evaluating sequence similarity, a continuous stretch of gaps (–) has been counted as one substitution regardless of its length. Filled arrowheads indicate identities, and open arrowheads conservative substitutions between the cGMP-gated channel and the cyclic AMP-binding domain of the *E. coli* CAP; the comparison is based on the sequence alignment of CAP with cGK (2) (ref. 23).

and the M_r estimated by SDS-PAGE¹² (63K) may be attributed to the inaccuracy inherent in the measurement of M_r of membrane proteins by the electrophoretic method²¹ and/or to proteolytic modification *in vivo* or during preparation of the channel.

Similarity matrix analysis²² of the cGMP-gated channel detects no extensive amino-acid sequence similarity to other ionic channels, membrane transporters and G-protein-coupled receptors of known sequence. The deduced amino-acid sequence of the cGMP-gated channel was analysed for local hydrophobicity and predicted secondary structure (Fig. 3). There are six hydrophobic segments with predicted secondary structure (referred to as H1–H6). These segments comprise ~20 amino-acid residues. Segments H4 and H5 probably represent transmembrane α -helices. Some or all of the four remaining segments may also span or interact with the membrane (see below). Segments H2, H3, H5 and H6 contain one to four charged (mostly negative) residues and segment H4, one histidine residue. In segments H2 and H5, the charged and polar side chains are clustered mainly on one side of the α -helix.

The region comprising 80 amino-acid residues located on the C-terminal side of segment H6 shows significant amino-acid-sequence similarity to the two tandem cGMP-binding domains of cGMP-dependent protein kinase²³ (Fig. 4), segments of which are similar in sequence to the regulatory subunits of cAMP-dependent protein kinase and the *Escherichia coli* catabolite gene activator protein (CAP)^{23,24}. The dimeric crystal structure of CAP with two bound molecules of AMP has been determined²⁵. By analogy with CAP, the side chain of Arg 559 of the cGMP-gated channel may interact with the phosphate group, and the side chain of Glu 544 may interact with the 2'-OH group of the ribose ring of cGMP. Moreover, glycines 508, 520, 539 and 543 are conserved in cGMP-dependent protein kinase as well as in CAP, and each of the corresponding glycines in CAP is located at the end of a β -strand or in a bend between two β -strands. Thus, it is suggested that these amino acids are important for the correct formation of the cGMP-binding pocket. The putative cGMP-binding region of the cGMP-gated channel is partly hydrophobic, which may indicate that a portion of the binding pocket is buried in the membrane. The region preceding segment H1 contains the consensus sequence Asn-Lys-X-Asp (residues 119–122), where X can be any amino acid, for binding the guanine ring of GTP/GDP (ref. 26), although these amino-acid residues are part of a long sequence composed of mostly Lys, Glu and Asp residues. It is possible that these residues may also be involved in the cGMP-binding site.

The relatively low hydrophobicity of segments H1, H2, H3 and H6 makes it difficult to predict the number of transmembrane segments and thus the transmembrane topography of the cGMP-gated channel. It is reasonable to assign the putative cGMP-binding region, located on the C-terminal side of segment H6, to the cytoplasmic side of the membrane. The cGMP-gated channel does not possess a hydrophobic N-terminal sequence indicative of a typical signal sequence. On the assumption that the N terminus is located on the cytoplasmic side, there should be an even number of transmembrane segments. By analogy

with other channels of known sequence, we favour the view that the cGMP-gated channel has either six (like the potassium channel²⁷ or one repeat of the sodium channel²⁸ and the calcium channel^{29,30}) or four transmembrane segments (like the ryanodine receptor³¹ or subunits of neurotransmitter-gated ionic channels^{28,32}), as schematically shown in Fig. 5a, b. The possibility that there are only two transmembrane segments (probably H4 and H5) cannot be excluded. The proposed models are consistent with one of the five potential N-glycosylation sites (Asn 423 for the model in Fig. 5a or Asn 327 for the model in Fig. 5b) being located on the extracellular side of the membrane; the remaining potential sites are asparagines 90, 91 and 177.

Functional expression

To examine whether the cloned cDNA actually encodes a functional cGMP-gated channel, we performed expression studies. The cDNA, including the poly(dA) tract, was linked with the bacteriophage SP6 promoter and transcribed *in vitro* with SP6 polymerase. The resulting mRNA was injected into *Xenopus* oocytes, which were incubated for 2–3 days before being tested.

Inside-out patches of large diameter³³ excised from injected *Xenopus* oocytes showed a large cGMP-activated outward current (membrane voltage V of +50 mV; Fig. 6a). The cGMP-activated current was reversible and its amplitude remained

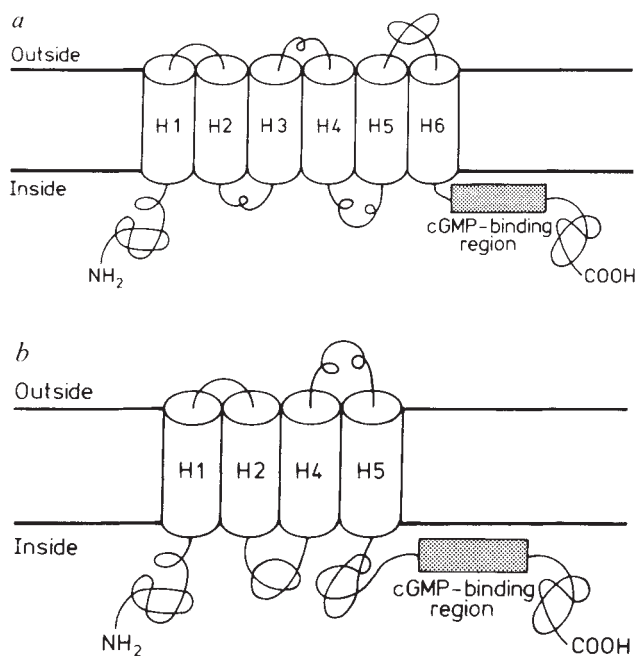


FIG. 5 Proposed transmembrane topography of the cGMP-gated channel. The presence of six (a) or four (b) putative transmembrane segments (indicated by cylinders) is assumed, which are displayed linearly; in b, it is possible that H3 and/or H6, instead of H1 and/or H2, span the membrane. The putative cGMP-binding region is also shown.

constant for several seconds, suggesting that the channel does not desensitize or inactivate in the presence of the agonist cGMP. All the 16 oocytes tested (5 injections, 20 patches) except one showed cGMP-activated currents, maximal amplitudes at saturating cGMP concentrations ($\geq 200 \mu\text{M}$) being 2–5 nA. No such response to cGMP was detected in three non-injected oocytes (5 patches) and in two oocytes (7 patches) that had been injected with mRNA specific for a potassium channel³⁴. The cGMP-activated current was reversibly blocked to one half by $\sim 40 \mu\text{M}$ *l*-cis-diltiazem (data not shown).

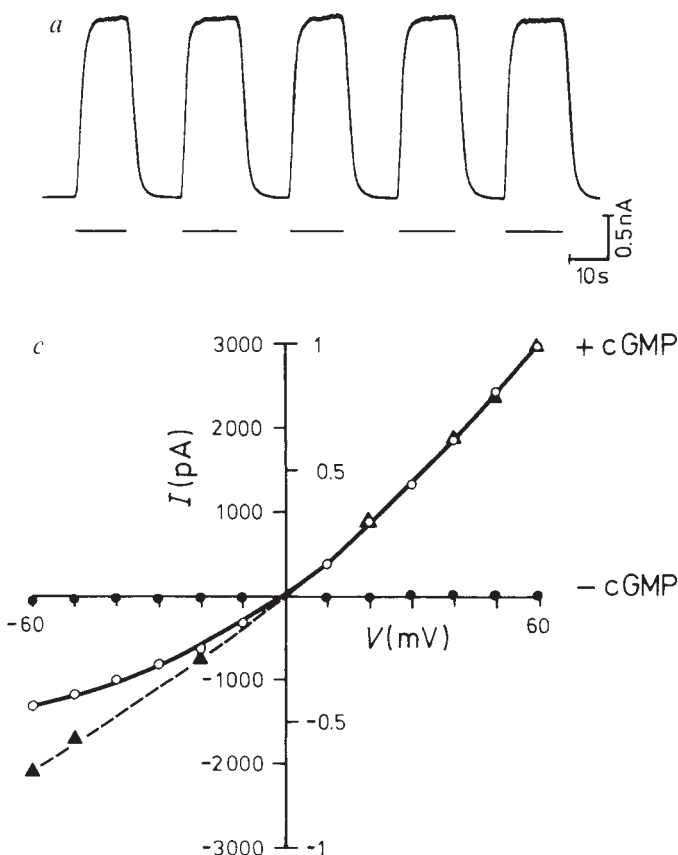


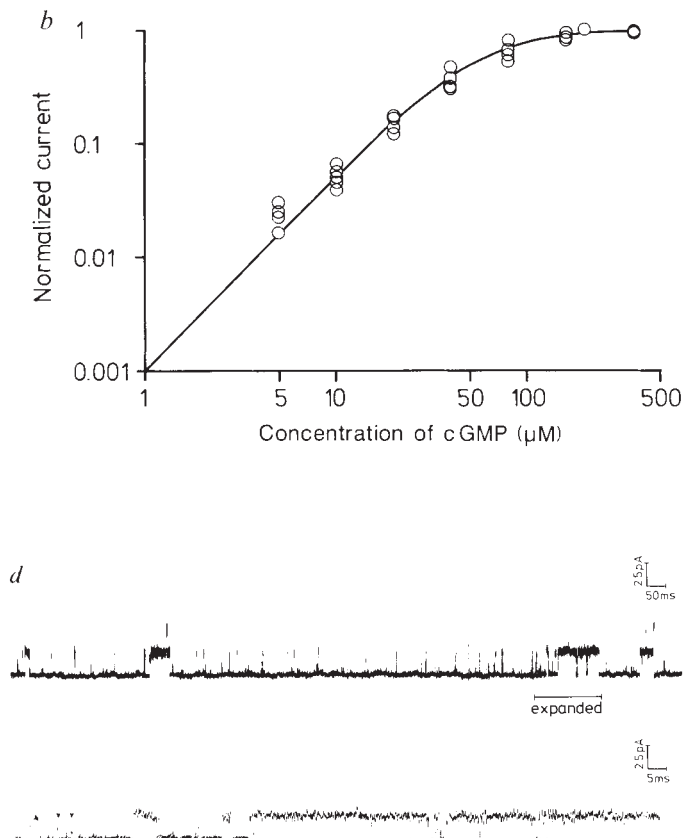
FIG. 6 Properties of the cGMP-gated channel expressed in *Xenopus* oocytes injected with mRNA derived from the cloned cDNA. *a*, Macroscopic currents activated by $200 \mu\text{M}$ cGMP. Inside-out patch. Membrane voltage (V) = $+50 \text{ mV}$. The duration of perfusion with cGMP is indicated by bars. *b*, cGMP-dependence of the current amplitudes normalized from five experiments in double logarithmic coordinates. The solid line has been drawn according to the equation in the text with $K_{1/2} = 52.3 \mu\text{M}$ and $n = 1.75$ ($V = +50 \text{ mV}$ in four experiments and $V = -40 \text{ mV}$ in one experiment). *c*, Current-voltage relation of the cGMP-gated channel. Macroscopic current in the presence (open circles) and absence (filled circles) of $200 \mu\text{M}$ cGMP; single-channel current in the presence of $5 \mu\text{M}$ cGMP (filled triangles). Numbers on the left-hand side of the ordinate refer to macroscopic currents, and numbers on the right-hand side to single-channel currents. *d*, Traces of single-channel currents activated by $5 \mu\text{M}$ cGMP at $V = +120 \text{ mV}$. Inside-out patch. Outward current upwards. Low-pass filtering at 2 kHz (-3 dB). Mean amplitudes of single-channel currents from two experiments were $2.38 \pm 0.21 \text{ pA}$ and $2.48 \pm 0.22 \text{ pA}$ (mean $\pm$ s.d.; from ~ 800 and $\sim 1,200$ single-channel events, respectively). The lower trace represents an expanded form of the current trace indicated by a bar. At least two classes of elementary currents are observed; smaller current events are indicated by arrowheads.

METHODS. The pSP65 recombinant (pRCG1) carrying the complete protein-coding sequence for the cGMP-gated channel was constructed as follows. The 457-base-pair (bp) *Nde*I(2,332)/*Nde*I (vector) fragment derived from clone pCG24 was cleaved by *Pvu*II, and the resulting 227-bp *Nde*I(2,332)/*Pvu*II(vector) fragment was ligated with the *Hpa*I(268)/*Nde*I(2,332) fragment from pCG24. The 2,292-bp ligation product was isolated by agarose (1%) gel electrophoresis and cloned into the *Hinc*II site of pSP65 in the same orientation as the SP6 promoter to yield pSP65CG.

Figure 6b shows dose-response relations for the cGMP-activated macroscopic current from five different patches. The solid curve was calculated according to:

$$I/I_{\max} = C^n / (C^n + K_{1/2}^n)$$

where $I/I_{\max}$ is the normalized current amplitude, C the cGMP concentration, $K_{1/2}$ the concentration of cGMP at which the current amplitude is half-maximal, and n the Hill coefficient ($K_{1/2} = 52.3 \mu\text{M}$; range 43–71 μM and $n = 1.75$; range 1.5–2.1). This result indicates that the channel is cooperatively activated



Finally the *Rsa*I (–176)/*Hpa*I(268) fragment from pCG101 was cloned into the *Hinc*II site of pSP65CG to yield pRCG1. mRNA specific for the cGMP-gated channel was synthesized *in vitro*⁴⁸, using *Hind*III-cleaved pRCG1 as template; transcription was primed⁴⁹ with the cap dinucleotide $m^7\text{G}(5')\text{ppp}(5')\text{G}$ (0.5 mM). The size (2.8 kb) of the mRNA, estimated by agarose (1.2%) gel electrophoresis, agreed with that expected from the structure of the plasmid. The mRNA was injected into *Xenopus laevis* oocytes (mRNA concentration, $0.4 \mu\text{g} \mu\text{l}^{-1}$; average volume injected per oocyte, $\sim 50 \text{ nl}$). Macroscopic and single-channel current measurements on excised inside-out patches³³ were made after incubation³³ of injected oocytes for 2–3 days, followed by removal of the follicular cell layer and the vitelline membrane⁵⁰. The solution in the pipette and the perfusion medium contained (in mM): 100 KCl, 10 EGTA-KOH and 10 HEPES-KOH (pH 7.2). For determination of relative ion permeabilities from the reversal voltage (V_{rev}) under bilionic conditions, KCl in the pipette solution was replaced by NaCl, and KCl in the perfusion medium by the respective ion species. In both solutions, HEPES-KOH buffer was replaced by Tris-HCl buffer, and the EGTA concentration was lowered to 1 mM. Measurements *in situ* on excised patches of isolated bovine rod outer segments confirmed that replacement of HEPES-KOH by Tris-HCl did not affect the cGMP-stimulated membrane current (H. Lühning, unpublished observation). Junction potentials varied between $+2.0$ and -1.9 mV and were neglected. cGMP-containing solution was applied by pressure through a glass pipette in front of the patch pipette. Macroscopic currents were either acquired on-line using a PDP 11/73 computer or stored on magnetic tape for off-line analysis. Single-channel currents were filtered at 2 kHz (-3 dB), stored on magnetic tape and analysed off-line on an Atari personal computer using standard single-channel analysis programs (Instrutech, Mineola, New York).

by cGMP. The $K_{1/2}$ and n values are similar to those observed *in situ* in amphibian^{4,10,11} and mammalian⁸ rod photoreceptors and cAMP failed to activate the channel at 1 mM concentration.

In the absence of divalent cations the I - V relation of the macroscopic current was slightly outward rectifying (Fig. 6c, open circles) and closely matches the I - V relation observed in excised patches from amphibian³⁵ and mammalian⁸ rod photoreceptors under similar conditions. The leak current recorded in the absence of cGMP was negligible (filled circles). The difference in voltage dependence between the macroscopic current (open circles) and the single-channel current (filled triangles) was small and became noticeable only at negative voltages. The small difference may be attributed to a weak voltage-dependence of gating and is in agreement with the observations *in situ*^{10,11} and in the reconstituted system¹⁴.

Figure 6d shows single-channel currents ($V = +120$ mV) recorded in the absence of divalent cations and at a low cGMP concentration. The mean current amplitude in 100 mM KCl was 2.4 pA corresponding to a single-channel conductance of 20 pS, but smaller conductance sublevels (8–10 pS) were also observed. The single-channel conductance observed *in situ*^{10,11} is 25 pS and that in the reconstituted system¹⁴ is 26 pS. At the membrane voltage used, periods with brief channel openings (mean open time $\tau_0 \sim 1$ ms) were interrupted by much longer openings ($\tau_0 \geq 10$ ms), indicating that the time distribution of opening is not uniform and may be described by at least two exponential time constants.

The ion selectivity of the expressed cGMP-gated channel was examined by measurement of the reversal voltage V_{rev} under symmetrical biionic conditions. The following values for V_{rev} were obtained (mean $\pm$ s.d.; n indicating the number of experiments): NH_4^+ , $+27.3 \pm 1.7$ mV ($n = 3$); K^+ , $+0.35 \pm 1.3$ mV ($n = 3$); Li^+ , -11.6 ± 1.7 mV ($n = 5$); Rb^+ , -14.6 ± 2.5 mV ($n = 5$); Cs^+ , -25.6 ± 1.6 mV ($n = 4$). Permeability ratios P_i/P_{Na} calculated from V_{rev} according to the Goldman-Hodgkin-Katz equation yielded the following series of ion selectivity:

$$\begin{aligned} \text{NH}_4^+ > \text{K}^+ \sim \text{Na}^+ > \text{Li}^+ > \text{Rb}^+ > \text{Cs}^+ \\ = 2.93:1.01:1:0.63:0.56:0.37 \end{aligned}$$

Similar relative ion permeabilities have been determined in excised patches from amphibian^{1,5-7} and mammalian⁸ rod photoreceptors.

Discussion

The primary structure of the rod photoreceptor cGMP-gated channel has been deduced by cloning and sequencing the cDNA. The predicted structure suggests the presence of multiple transmembrane segments, which may be involved in forming the channel portion. On the carboxyl side of the putative transmembrane region, there exists a segment of 80 amino acids which exhibits significant amino-acid sequence similarity to the cyclic nucleotide-binding regions of cAMP-binding proteins and in particular of cGMP-dependent protein kinase. This strongly suggests that the corresponding region of the cGMP-gated channel is also involved in binding of cGMP. The channel is cooperatively activated by at least three^{10,11}, probably more^{3,12,13} cGMP molecules. Moreover, cGMP-dependent protein kinase which is cooperatively stimulated³⁶ by two cGMP molecules per monomer³⁷, contains two tandem homologous cGMP-binding regions, whereas the cGMP-gated channel contains only one such region. This indicates that the cGMP-gated channel is a homo-oligomeric complex, each constituent polypeptide having a single cGMP-binding site.

The expression of cGMP-gated channel activity by injection of *Xenopus* oocytes with specific mRNA derived from the cloned cDNA identifies the encoded polypeptide as the cGMP-gated channel protein of mammalian rod photoreceptors and suggests that this polypeptide alone is sufficient to form the functional channel with properties similar to those observed *in situ*. In particular the expressed channel is cooperatively activated by cGMP with a $K_{1/2}$ of several tens of micromolar; it represents a channel that does not discriminate very well between alkali cations and whose major single-channel conductance is 20 pS; the macroscopic cGMP-activated current exhibits a weak outward directed rectification, probably due to the weak voltage-dependence of gating and the almost linear I - V relation of the single channel; finally, the channel is blocked by the drug *l*-cis-diltiazem. □

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Coordinate expression of the murine *Hox-5* complex homoeobox-containing genes during limb pattern formation

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The homoeobox-containing genes of the *Hox-5* complex are expressed in different but overlapping domains in limbs during murine development. The more 5' the position of these genes in the complex, the later and more distal is their expression. Antero-posterior differences are also observed. A model is proposed that accounts for the establishment of these expression domains in relation to the existence of a morphogen released by the zone of polarizing activity. Comparison of these observations with the expression patterns of the genes of *Hox* complexes in the early embryo suggests that similar molecular mechanisms are involved in the positional signalling along the axes of both the embryonic trunk and the fetal limbs.

position in the complex: the more 5' a gene, the more distally restricted is its expression domain. We present evidence for a sequential appearance of the transcripts in early stages of limb bud outgrowth, taking place in cells from the postero-dorsal pole of the limb. In addition, each gene shows a graded transcript distribution along the A-P axis, which is maximal at the posterior margin and progressively decreases anteriorly. These shifted and partially overlapping expression domains, as well as later regional changes in the abundance of the RNAs, led us to propose that a key step in establishing positional information involves precise combinations of gene products of the *Hox-5* complex in mesodermal cells of the developing limb. We propose a model that accounts for the establishment of the observed plurimolecular gradient and which is based on the existence of a primary diffusible signal in the form of a concentration gradient set up by the ZPA.

Sequential activation of *Hox-5* genes

The mouse forelimb bud becomes apparent in early gestational day 9 as an outgrowth of the mesoderm underlying the anterior portion of an initial lateral epiblastic ectoderm thickening, the Wolffian ridge^{15,16}. A comparative study of early day-9 post coital (p.c.) embryos revealed the presence of *Hox-5.1*, *-5.2*, *-5.3* and *-5.5* transcripts (Fig. 1a, both bright-field views). *Hox-5.1* was expressed throughout the entire forelimb bud mesoderm and in the adjacent axial mesoderm and neural tube. In fact, the forelimb field was included in the overall *Hox-5.1* expression domain, which extended in the trunk up to the second cervical vertebra by day 12.5 p.c. *Hox-5.2* was strongly expressed in the limb bud mesoderm, with a sharp expression boundary. The expression domain of *Hox-5.3* was similar to that of *Hox-5.2* except in very anterior regions of the forelimb buds, where the *Hox-5.3* transcripts became distally restricted (Fig. 1a, left bright-field view). Consequently, the anterior-most areas of the limb buds in this plane of section were still positive for the *Hox-5.2*-specific probe, but negative for the *Hox-5.3*-specific probe. By contrast, *Hox-5.5* was expressed very weakly, and specifically in the dorsal half of the post-axial (posterior) limb mesoderm (Fig. 1a, right bright-field view). *Hox-5.6* transcripts were not detected in the 9.25-day p.c. forelimb bud. Half a day later (9.75 days p.c.), however, *Hox-5.6* transcripts appeared in the buds, but only in very posterior planes of section with a preferential dorsal distribution (Fig. 1b). Therefore the transcription of the *Hox-5.6* gene is activated in early stages of forelimb bud development, between 9.25 and 9.75 days p.c. in a spatially restricted domain, spanning preferentially postero-dorsal forelimb bud mesoderm. This initial *Hox-5.6* expression domain was similar to that of *Hox-5.5* observed several hours earlier (9.25 days p.c.; Fig. 1a, right bright-field view). This suggests that homoeogene transcripts, at least those of *Hox-5.5* and *-5.6*, appear in the same discrete region of the forelimb bud, in a temporal sequence. Only the *Hox-5.1* and *-5.2* genes were expressed in the forelimb field before limb bud outgrowth (at 8.75 days p.c., data not shown). At a comparable stage of development, *Hox-5.3* and *-5.5* were expressed only in very

THE study of patterning and development of the vertebrate limbs is one of the most fascinating ways to investigate morphogenetic mechanisms. The establishment of the limb axes and consequently of pattern formation, is believed to be controlled mainly by two particular areas, the zone of polarizing activity (ZPA)¹⁻³ and the apical ectodermal ridge (AER)⁴. The ZPA, the putative source of a diffusible morphogen⁴⁻⁶, is located at the posterior margin of the limb bud and is involved in the specification of pattern along the antero-posterior (A-P) axis, whereas patterning along the proximo-distal (P-D) axis is controlled by interactions of the AER and the underlying mesenchymal cells^{7,8}. Little is known, however, about the molecular nature of the signalling mechanisms involved in such morphogenetic events. Accumulating evidence indicates that vertebrate homoeobox-containing genes could have a key role during limb regeneration⁹ and development¹⁰⁻¹². We previously reported that two genes of the mouse *Hox-5* complex (*Hox-5.2*, *Hox-5.3*) are strongly expressed during all stages of limb morphogenesis with the same cellular specificities but subtle differences in the extent of their domains of expression^{10,13}. We have also cloned and analysed two other genes in this complex, *Hox-5.5* and *Hox-5.6*, located upstream of (5' to) the *Hox-5.2* and *Hox-5.3* genes (J.-C.I.-B. *et al.*, manuscript in preparation). Here we report on an *in situ* hybridization analysis of the expression domains of five genes belonging to the *Hox-5* complex (*Hox-5.1*, *-5.2*, *-5.3*, *-5.5*, *-5.6*) throughout six stages of murine limb development, using antisense RNA probes specific for each of these homoeogenes. We show that with the exception of *Hox-5.1* (ref. 14), which is expressed in the whole limb and flank mesenchyme, each gene has a unique expression domain, precisely restricted along the P-D axis of the developing limbs according to its

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posterior regions of the trunk (data not shown). It is intriguing that the genes located at 5' positions in the *Hox-5* complex were expressed in the limb buds several hours after the expression of those located at more 3' positions. This strongly suggests that the timing of expression of genes of the *Hox* complexes, at least in the limb buds, depends on their position in the complexes according to the following rule: 3' → early; 5' → late.

Expression domains along the P-D axis

A constant feature of the expression of the *Hox-5*-complex genes throughout limb development is that each gene has a distinct P-D expression domain in the mesodermal cell component. The ectodermal layer, including the AER, was found to be negative

for all the probes specific for *Hox-5*-complex genes (Fig. 2). Only *Hox-5.1* did not have a proximal boundary of expression, because it was widely expressed in the adjacent axial tissues (Fig. 1a-c). By contrast, each of the four other genes of the *Hox-5* complex was expressed in the distal limb mesoderm, and showed a distinct proximal expression boundary. Thus *Hox-5.2* was expressed near the proximal part of the limb, whereas the expression of *Hox-5.6* was the most distally restricted (Fig. 1). These overlapping but shifted expression domains are best seen in the undifferentiated limb bud mesenchyme, as exemplified by longitudinal sections of 9.75-day p.c. (Fig. 1b) and 10.5-day p.c. (Fig. 1c) forelimbs. But the proximal expression boundaries were quite distinct in more anterior planes of section (for

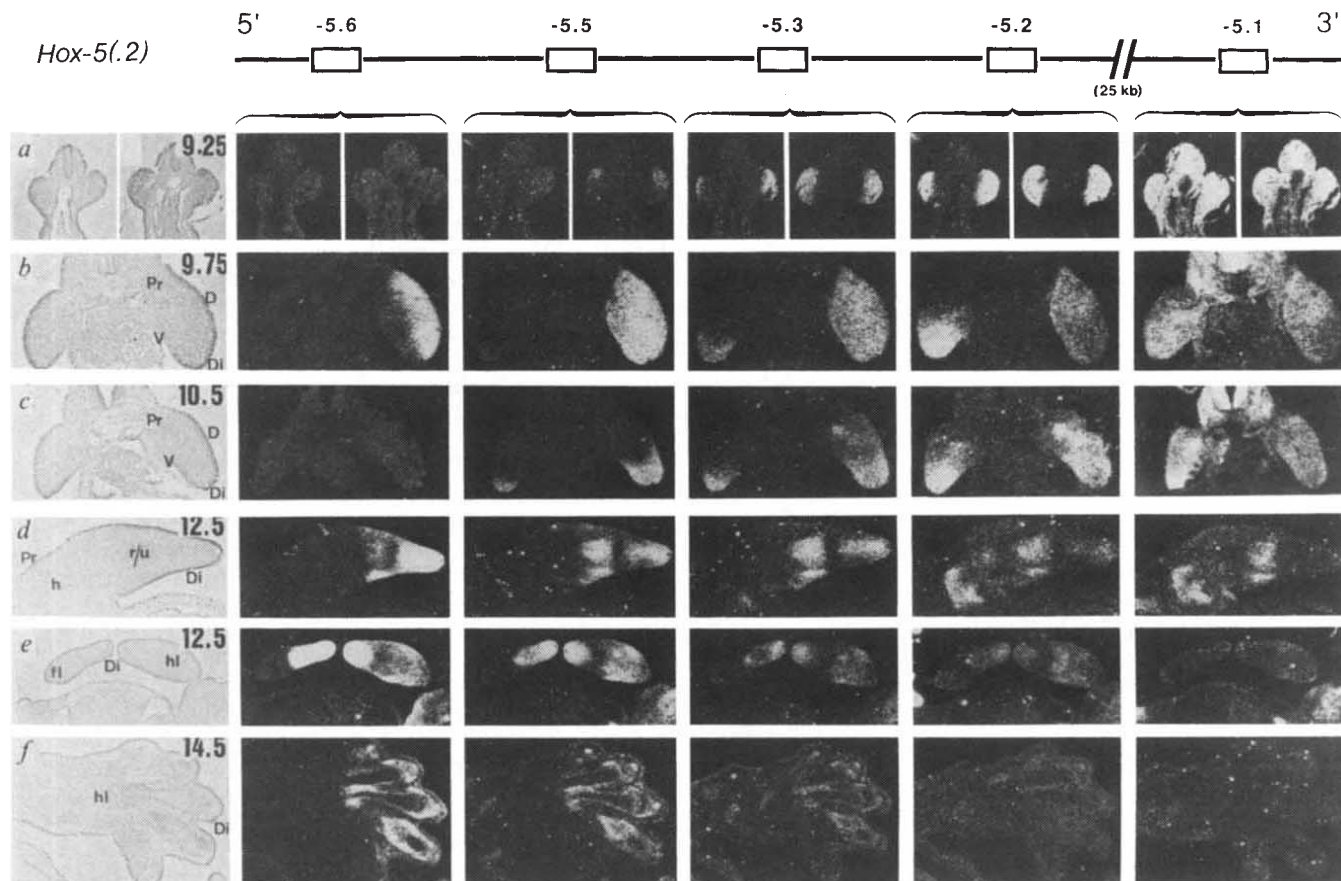


FIG. 1 Restriction of the expression domains of the *Hox-5*-complex genes along the P-D axis of the developing mouse limbs. The transcript domains of five genes of the *Hox-5* complex are shown as detected by *in situ* hybridization with specific probes, on neighbouring sections of fetal limbs at successive developmental stages. The left-most panels show bright-field views of the sections, and the panels to the right of those show dark-field views of neighbouring sections hybridized with probes for (from left to right) *Hox-5.6*, *-5.5*, *-5.3*, *-5.2* and *-5.1* transcripts. The corresponding alignment of these genes in the *Hox-5* complex on chromosome 2 is indicated (top), the overall distance being <100 kilobases (kb). a, Two transverse sections across the forelimb buds of a 9.25-day p.c. fetus. The section shown on the left is the anterior part of the bud, whereas that shown on the right is from a more posterior part. b, Longitudinal section through the forelimb buds of a 9.75-day p.c. fetus (the section is transverse to the body axis). The limb located on the left side is sectioned more anteriorly than the other. c, Longitudinal section through the forelimb buds of a 10.5-day p.c. fetus. Again, the left limb is sectioned more anteriorly. d, Longitudinal section through a 12.5-day-old forelimb. e, Longitudinal section through the extremities of a forelimb on the left, and of a hindlimb on the right, of the same 12.5-day p.c. fetus. f, Section through a 14.5-day p.c. hind foot. Abbreviations: hu, humeral blastema; r/u, radius/ulna blastema; fl, forelimb; hl, hindlimb; Pr, proximal; Di, distal; D, dorsal; V, ventral. Numbers in top-right corners of bright-field views refer to age of embryos or fetuses (days p.c.).

METHODS. The *Hox-5.5* and *Hox-5.6* genes were isolated during a 'walk' along the *Hox-5* gene complex. Details of their structures and expression will be published elsewhere (J.-C.I.-B. *et al.*, manuscript in preparation). ³⁵S-labelled antisense RNA probes were synthesized from DNA templates covering sequences 3' from the homeoboxes. The DNA templates used for *Hox-5.1*, *-5.2* and *-5.3* have been previously described^{13,14}. Embryo recovery, embedding, sectioning and *in situ* hybridization were performed as previously described¹⁰, except that the prehybridization step was omitted. Several embryos at day 9.25, 10, 10.5, and 12.5, and one 14.5 day old fetus, were analysed. More than 700 histological sections containing limbs were examined after hybridization to the five different probes (a total of ~140 sections for each probe), and all the sections showed concordant expression patterns. All the material was analysed in successive serial sections, allowing us to correctly orientate the specimen. A first orientation was made during embedding and sectioning and latter more precisely interpreted when looking at the full series (an example of this critical point is given in Fig. 3). Neighbouring sections from the same fetus were hybridized successively with the five different probes to exclude the possibility of differences arising because of the progression of the sectioning within the sample. The autoradiography exposures were identical for each experiment (14 days) and sections were photographed under a Zeiss SV8 stereomicroscope with identical exposure times for comparative dark-field views.

example, the left forelimb in Fig. 1*b*, which was cut more anteriorly, and both limbs in Fig. 1*c*), and became indistinguishable in the most posterior planes of sections (for example, the right limb bud in Fig. 1*b*; see below for a discussion of the A-P specificities). Similarly, distinct proximal expression boundaries were found in the undifferentiated hindlimb buds (data not shown). From these results we conclude that the ordering of the genes in the *Hox-5* complex not only reflects or determines the anteriority (posteriority) of their expression in the developing trunk and the timing of their transcription, but also the position of their domains of expression along the P-D axis of the growing limb buds. The expression of *Hox-5.2* and *-5.3* genes is confined by mid-gestation to central limb precartilaginous condensations¹⁰. Such a restriction was also observed for *Hox-5.1*, *-5.5* and *-5.6* genes (see, for example, Fig. 1*d*), the expression of the 5' genes (such as *Hox-5.5*, *-5.6*) being restricted to more distal blastemas. Thus, in the 12.5-day p.c. forelimb, *Hox-5.1* and *-5.2* are expressed strongly in the proximal humeral head blastema (Fig. 1*d*), and *Hox-5.3* only weakly, whereas *Hox-5.5* was expressed only in the radius/ulna blastema and distally. The expression patterns become more complex as limb growth proceeds, because a decrease of the hybridization signal intensity of 'proximal' genes (such as *Hox-5.2*) occurs progressively in distal regions. This decrease takes place in regions where more 'distal' gene (such as *Hox-5.5*) transcripts appear. For example, the *Hox-5.2* signal was weaker in the post-axial half of the 10-day p.c. forelimb tip, a region where *Hox-5.5* and *Hox-5.6* transcripts strongly accumulate (see Fig. 3 and Fig. 1*d*, *e* for similar observations at 12.5 days p.c.). This hierarchy in local transcripts abundancies still existed in the 14.5-day p.c. limb, where *Hox-5*-complex gene transcripts were present in the hindlimb extremity in mesenchymal cells surrounding the phalangeal cartilage anlage (Fig. 1*f*). Again, *Hox-5.6* transcripts were abundant, whereas the labelling *Hox-5.5* and *-5.3*, transcripts decreased, and was only that of background for *Hox-5.2* and *-5.1* (Fig. 1*f*).

P-A distribution of *Hox-5* transcripts

It is mentioned above that the onset of transcription of at least some of the *Hox-5*-complex genes occurs preferentially in the postero-dorsal zone of the limb bud. Later, when the limb bud tissue consists of undifferentiated mesenchyme (day 9.25 to day 10.5 p.c.), each gene of the *Hox-5* complex, with the exception of *Hox-5.1*, still shows a preferential expression in the posterior area so that the transcripts are unequally distributed along the A-P axis, with a maximal amount at the post-axial side. Because of the distinct P-D specificities, these A-P differences will be visible for different genes at different P-D levels. Figure 3 shows serial sections, regularly spaced, through the forelimb buds of a 10.0-day p.c. fetus in a nearly frontal plane, so that both A-P and P-D axes are visualized (as seen on the scheme *f*, the plane of section is very slightly dorso-ventral, but essentially antero-posterior). In very dorsal sections (Fig. 3*a*), a 3' gene (*Hox-5.2*) and a 5' gene (*Hox-5.5*) had quite similar expression domains in the post-axial mesoderm, whereas the most anterior mesoderm showed no expression of these genes. Sections taken more ventrally and towards the tip of the bud showed *Hox-5.2* expression in a wide part of the bud, with the very anterior margin remaining unlabelled (Fig. 3*b-d*). By contrast, *Hox-5.5* expression remained far more restricted to very posterior and distal cells (Fig. 3*b-d*; note that the posterior margin was labelled up to the base of the limb). In addition, there was a gradual decrease in the amount of *Hox-5.2* transcripts towards more distal regions, roughly corresponding to areas where *Hox-5.5* was expressed (Fig. 3*d-e*). The expression domains of the *Hox-5.2* to *-5.6* genes have been plotted on a single diagram of each section (Fig. 3, far-right panels), showing that the more 5' a gene is located in the *Hox-5* complex, the more its transcript domain is restricted posteriorly and distally. At this stage, a

given transcript domain fully overlaps that of the genes located more 3'.

Such A-P specificities are also seen on sagittal planes of section. The section of a 10.5-day p.c. forelimb shown in Fig. 4*a* is rather proximal. At this level, *Hox-5.2* was expressed in the whole mesoderm, although the signal was stronger in the central condensation. By contrast, *Hox-5.5* transcripts were restricted to the postaxial mesoderm as well as to along the ventral side. *Hox-5.6* expression is even more restricted to the very posterior cells. In more distal sections, *Hox-5.5* and *-5.6* transcripts progressively became distributed among the whole mesoderm (data not shown). A reminiscence of these A-P gradients persisted after cytodifferentiation and was visible by p.c. day 12.5 in a band of cells extending very proximally in the posterior parts of both hindlimbs and forelimbs (Fig. 4*b*, arrows).

Spatial distribution and positional information

Each gene of the *Hox-5* complex thus displays specific expression domains in the mesoderm of the developing limb buds. To facilitate a spatial view of these complex expression domains, Fig. 5 shows a schematic representation of the expressions domains of the *Hox-5.2* to *-5.6* genes along two axes of a gestational day-10 forelimb bud. The expression boundaries of each gene were deduced from experimental data and plotted on a single diagram where the overlaps are shown by darkness. These expression patterns are determined by three main features. First, the expression domains of the *Hox-5*-complex genes are shifted along the P-D axis. Second, there is a differential transcript distribution for the genes along the limb A-P axis resulting in the transcript abundance being maximal at the posterior side and decreasing anteriorly. Thus, the proximal expression boundary of each gene will not be perpendicular to the P-D axis, but rather proximal posteriorly and displaced distally in more anterior regions (Fig. 5*a*; see also Fig. 3). Third, the expression domains are regulated along the limb dorso-ventral axis in a two-step mechanism: initially, transcripts appear in the postero-dorsal mesoderm (for example, *Hox-5.6* in Fig. 5*b*); later, the expression becomes more widespread along the postero-ventral side of the limb.

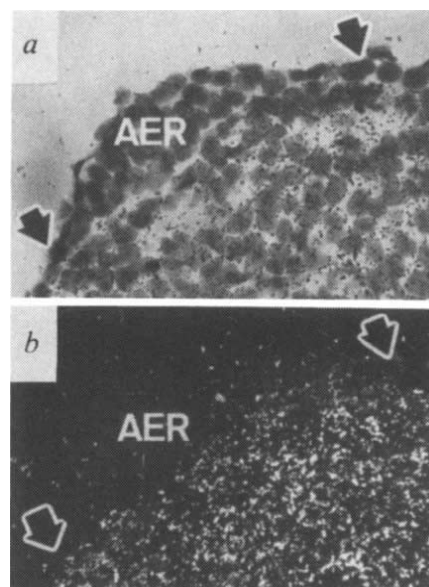
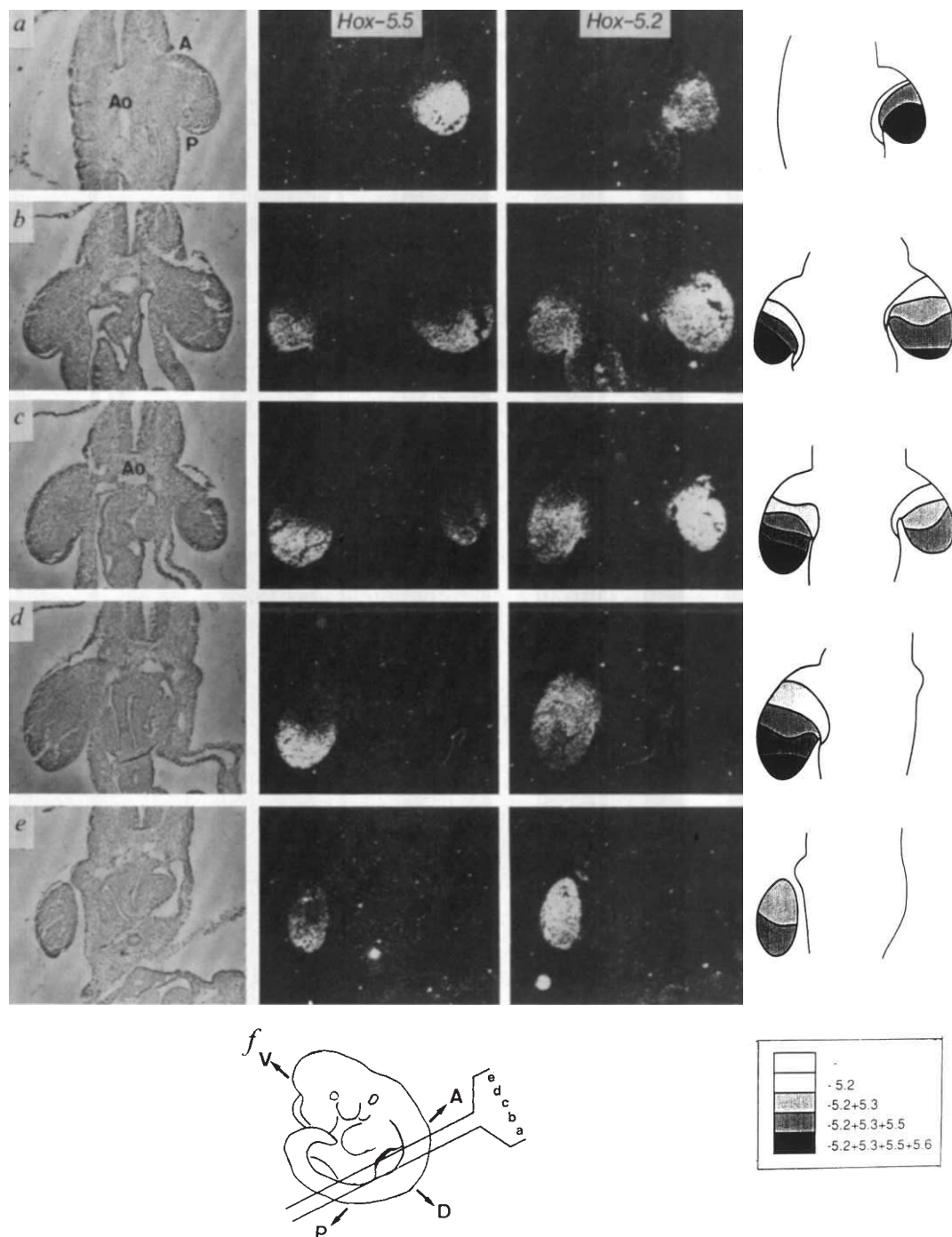


FIG. 2 The expression of the *Hox-5*-complex gene transcripts is restricted to mesoderm-derived cells. This picture shows high-power magnification of a distal part of a 9.75-day p.c. forelimb section hybridized to the *Hox-5.2* probe and visualized in bright field (*a*) or dark field (*b*). The grains are found exclusively in the mesenchymal cells, whereas the AER as well as the ectodermal layer (arrows) are negative for the *Hox-5.2* probe. Comparable results are obtained with the other probes specific for *Hox-5*-complex genes.

As a result of such transcript distributions, one area of the developing limb bud will contain all five different *Hox-5*-complex gene transcripts. This zone of maximum overlap is located at the posterior side of the limb bud and therefore appears to coincide with the region containing the ZPA¹⁻³. This polarizing region is capable, when applied in an anterior position, of inducing mirror-image duplication of the chick wing skeleton² and is considered as a source of a diffusible morphogen²⁻⁶. Considering these properties, we suggest one possible way by which such transcript patterns could be generated. Two

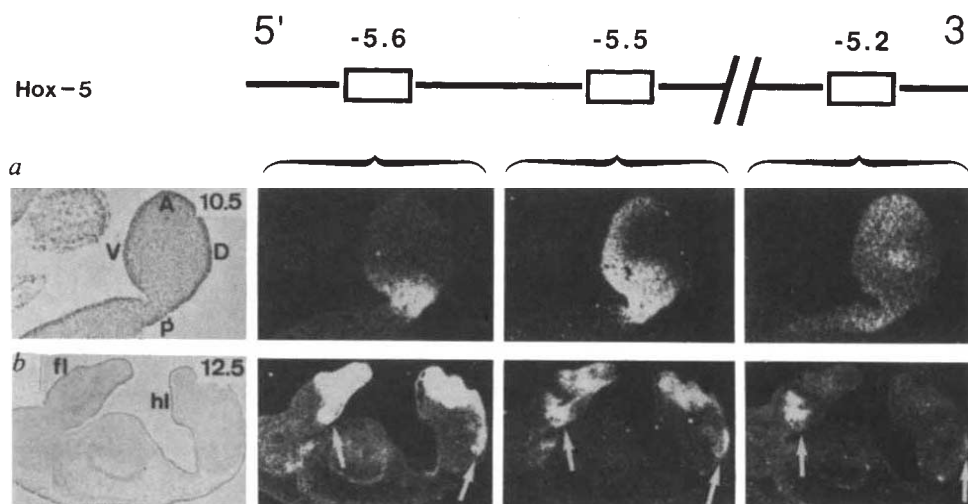
basic assumptions are required. First, that the ZPA, located at the posterior margin of the progress zone, releases a morphogen M having a concentration gradient in the limb bud that is maximal in the ZPA. M has a positive, stable and linear effect on the amount of all *Hox-5*-complex gene transcripts but exclusively in the regions where the concentration of M is above a threshold level. This positive effect could be mediated, for example by transcriptional activation or messenger RNA stabilization. Second, each one of the *Hox-5*-complex genes can be 'open' or 'closed for business' (that is, for transcription) as

FIG. 3 Spatial distribution (far-left bright-field view and adjacent two dark-field views) of *Hox-5*-complex gene transcripts in 10-day p.c. forelimbs. *a-f*, Different serial sections (spacing 80 μ m) through the forelimb of a 10.0-day p.c. fetus. These serial sections were hybridized with the five different probes but, for clarity, only the results obtained with a 5' gene (*Hox-5.5*) and a 3' gene (*Hox-5.2*) are shown. The corresponding patterns of expression of four genes (*Hox-5.2* to *-5.6*) are summarized in the far-right panels. The boundaries of the four genes are shown by the intensity of grey indicating the degree of overlap of expression domains. The corresponding scale is shown at the bottom right. The progression of the sectioning (*a* through to *e*), is indicated on the schematic embryo shown in *f*. This shows that the planes of section do not exactly correspond to the limb A-P axis as indicated with arrows, but are slightly dorsal in the anterior part and slightly ventral in the posterior part. This plane of section is nevertheless shown as A-P in *a*. A very dorsal section is shown in *a* and the sectioning progresses towards more ventral and distal parts; *f* also shows that the axes of the limb do not always correspond to the axis of the body, which is curved, and therefore demonstrates the need of serial sections for a correct orientation (see, for example, the aorta or the dorsal part of the coelomic cavity, which are cut longitudinally in *a*, but clearly through the dorso-ventral axis in more ventral planes of section such as those of *c* or *d*). If the *Hox-5.5* and *-5.2* expression domains are very comparable in dorsal sections (spanning the post-axial mesoderm up to the proximal part, but negative in the anterior-most regions (*a*)), they become different in more ventral (distal) sections where *Hox-5.5*



expression is now restricted to a distal part but still expressed proximally in posterior areas. This series of schemes clearly shows the different boundaries observed along the A-P axis. A, anterior; P, posterior; V, ventral; D, dorsal; Ao, aorta.

FIG. 4 P-A distribution of *Hox-5*-complex gene transcripts in developing limb mesenchyme. *a*, Sagittal section of the proximal part of a 10.5-day p.c. forelimb. The panels are (from left to right), bright-field view, and dark-field views of neighbouring sections hybridized to the *Hox-5.6*, *-5.5* and *-5.2* probes. *b*, Sagittal plane of section crossing both forelimb and hindlimb of 12.5-day p.c. fetus. Note the posterior cells (arrows) which express *Hox-5.5* and *-5.6* in both limbs, up to very proximal levels. A, anterior; P, posterior; V, ventral; D, dorsal; fl, forelimb; hl, hindlimb. Corresponding alignment of genes in *Hox-5* complex shown (top), as is age of fetuses (top right of bright-field views; day p.c.).



proposed for an adaption of Lewis's model for the regulation of expression of homoeotic genes during the development of the *Drosophila*¹⁷⁻¹⁹. Initially, only *Hox-5.2* is open for business and therefore transcribed (or stabilized) in the flank mesoderm including the prospective limb fields. After limb bud outgrowth, a time-dependent mechanism will successively turn the neighbouring (5'-located) genes to the open for transcription state. Hence at the time when a given gene becomes open for transcription, its transcription will be activated, or its transcripts stabilized, only in the region where the local concentration of M is above threshold, and to an extent directly related to this concentration. Because the limb is growing in size, the ZPA moves towards a more distal position, and the areas where the concentration of M is greater than the threshold level are therefore displaced distally. This model integrates the time as a crucial parameter, because the balance between the time required for limb bud growth and the sequential opening of the *Hox-5* genes seems to be decisive (see Fig. 6 for a scheme of this discussion). The timing of this mechanism could be set up by the AER, which would therefore essentially regulate limb growth and thus allow the ZPA to move on distally. This model would give rise to expression patterns of genes of the *Hox-5* complex compar-

able to those that were observed and schematized above. The mechanism by which the genes are turned into an open state could be independent of *cis*-regulation in the *Hox-5* complex: it could for example rely on chromatin unwinding. Alternatively, a homoeoprotein could accumulate and cross-regulate the transcription of the 5' neighbouring genes, allowing it to respond to the presence of M in the correct areas. It is of interest that among the four considered genes, only the most 3'-located, *Hox-5.2*, was expressed in the flank mesenchyme before limb buds outgrowth (p.c. day 8.5). This expression in the mesoderm underlying the Wolffian ridge, which is a more extended domain than the forelimb and hindlimb fields, could make *Hox-5.2* competent for an early response to the ZPA regulatory effect as soon as M is released. Therefore, *Hox-5.2* function could be to provide an initial 'proximal' landmark to those cells engaged in forming the limb buds.

Various theoretical models that account for pattern formation in the chick limb have been proposed (for example, see refs 15, 20). Wolpert first suggested that positional information is provided by a concentration gradient of a positional signal²¹. The necessary information could be given by two gradients along the P-D and A-P axes, the latter one arising from the ZPA.

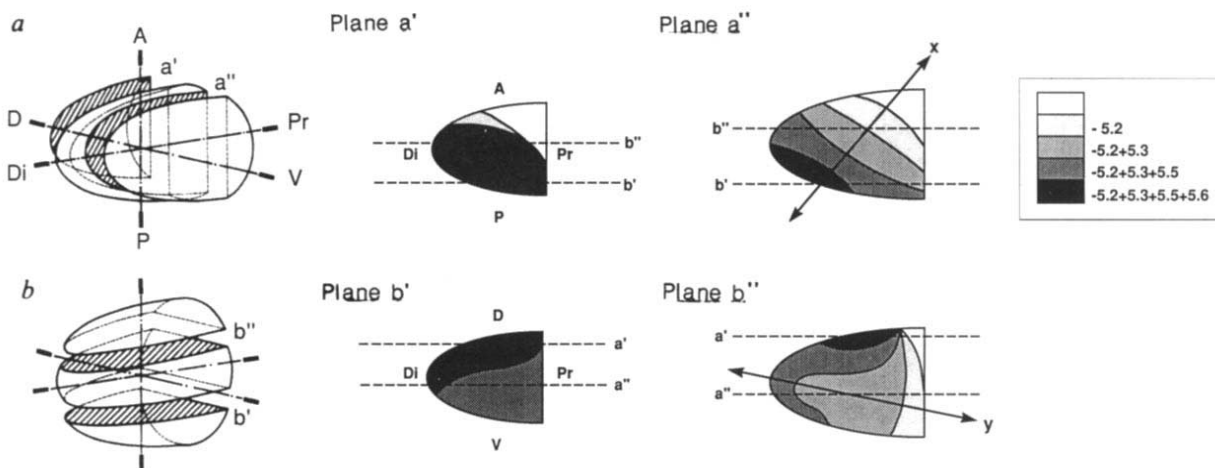


FIG. 5 Schematic representation of the expression domains of the *Hox-5.2*-*Hox-5.6* genes in the forelimb during gestational day 11. The expression domains are drawn according to two perpendicular planes of section, indicated under *a* and *b*, each of them showing two parallel sections shifted along the considered axis. *a*, Frontal sections in a very dorsal (*a'*) or slightly ventral (*a''*) plane. *b*, Longitudinal sections in a very posterior (*b'*) or slightly anterior (*b''*) plane. Different intensities of grey have been assigned to each gene from clear (*Hox-5.2*) to dark (*Hox-5.6*) in the same way as for Fig. 3.

A given intensity indicates the expression of the corresponding gene in addition to that of more 3' genes. The positions of the sections *a'* and *a''* are shown in the schemes of the sections *b'* and *b''* and vice versa. The expression boundaries are deduced from experimental data and correspond to the limit where the hybridization signal of a given gene falls to background level. The *x* and *y* axes are arbitrary and show the sequence of overlaps along two different axes (see the text). A, anterior; P, posterior; D, dorsal; V, ventral; Pr, proximal; Di, distal.

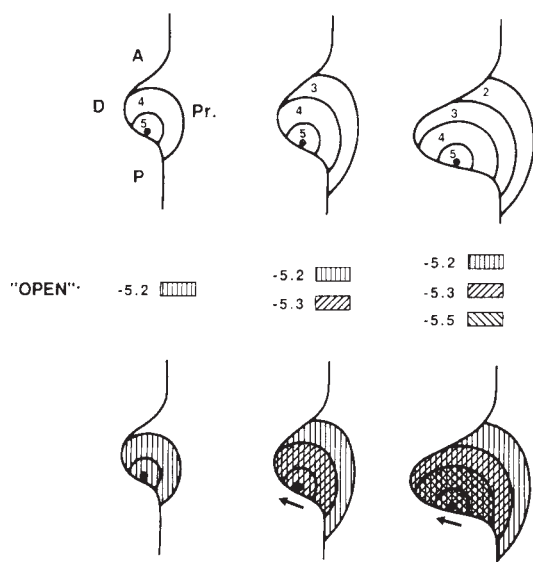


FIG. 6 Discussion scheme. This hypothetical scheme illustrates the time-dependent successive 'opening' of the *Hox-5*-complex genes during limb-bud outgrowth, leading to a more distal expression of the genes expressed later (or located 5'; see the text for details).

Alternatively, the standing-wave pattern model²² proposes the existence of a unique source of morphogen released from the ZPA. Our results now indicate that the *Hox-5*-complex homoeogene products could be necessary 'positional cues' in the developing mouse limbs, and that both A-P and P-D axes of the limb could be specified through a common mechanism involving a single gradient of positioning signal arising from the ZPA. This initial gradient would be translated into a plurimolecular gradient consisting of specific combinations of a discrete number of nucleoproteins encoded by genes of the *Hox-5* complex in each mesodermal cell of the limb. The combination of these different overlapping domains is shown in Fig. 5, where both the *x* and *y* axes (arbitrarily designed but corresponding roughly to the A-P and P-D axes) are shown to go through various zones of overlaps. A-P specification would rely on the overall amount of homoeoproteins present in a given cell, whereas P-D information would depend on the relative amount the products of the *Hox-5*-complex genes or on the amount of the principal protein present in the cell, or on both. This is consistent with previously published results showing that the *Hox-5.2* gene product forms a gradient which is maximal in a posterior region, possibly corresponding to the ZPA¹¹. Proteins encoded by genes of *Hox* complexes are probably transcription factors acting through their binding to DNA target

sequences (see, for example, refs 23, 24). Specific combinations of such factors could therefore differentially affect the transcription of putative target genes.

Several lines of evidence suggest that retinoic acid is the morphogen released by the chick ZPA^{5,6}. In the mouse, limb defects have been correlated with high enrichment of retinoic acid in the limb buds of fetuses exposed to retinoic acid²⁵. Three murine genes encoding retinoic acid receptors (RAR) have been cloned²⁶ (α -, β - and γ -RAR) and two of them, α - and γ -RAR, are homogeneously expressed in developing limb buds²⁷. In addition, RAR γ shows subsequent restriction to precartilaginous blastemas in a way similar to the expression of *Hox-5*-complex genes. Thus retinoic acid, through its binding to a receptor, could regulate the transcription of the *Hox-5*-complex genes, leading to limb pattern formation.

Conclusion

The expression scheme of the *Hox-5*-complex homoeogenes in the developing limbs strongly parallels that observed for the gene members of the *Hox* complexes along the A-P axis of the mouse embryo^{13,28-31}. This probably reflects at the molecular level a conservation of the mechanism by which positional cues are given, by means of homoeogene products, to the newly formed axes of the vertebrate body. A common mechanism could be involved in sequentially establishing the homoeogene expression domains along the embryonic and limb axes. Such a uniformity of signalling mechanisms has been previously suggested by Hornbruch and Wolpert, who showed that Hensen's node, a crucial area during gastrulation, does have polarizing activity when grafted onto a chick limb bud³². It has been suggested that determination of the mesoderm occurs at the time of the invagination through the primitive streak (for example, see ref. 33). Homoeogenes have been shown to be transcribed during gastrulation^{34,35} and were postulated to play a part during this period in setting up positional signalling along the A-P axis (discussed by Gaunt³⁴). Hensen's node could therefore be a key zone in the establishment of the expression domains of the genes of the *Hox* complexes along the rostro-caudal axis in a way similar to that of the ZPA in the context of limb development. At least, it is striking that Hensen's node, by partially regressing towards a more posterior position, goes in the direction of the more 'posterior' genes (those located 5' in the complex and therefore expressed posteriorly in the developing trunk), as does the ZPA when progressing towards a more distal part of the limb, in the direction of the 'distal' genes (those located 5' in the complex and therefore expressed distally during limb development). Because so far no other vertebrate *Hox* gene complex has shown similar features, the *Hox-5* complex (or part of it) could specify limb bud as well as specifying positional cues along the developing rostro-caudal axis. □

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X-ray time variations from Cygnus X-1 and implications for the accretion process

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THE binary system Cygnus X-1 is thought to contain a black hole that accretes matter from an O-type supergiant companion. Gravitational energy of the matter in the accretion disk is transformed to thermal energy, resulting in the emission of X-rays. These are emitted in successive bursts ('shots')¹. Here we demonstrate the existence of a time delay between X-rays in different energy ranges; this is manifest as a double-peaked structure in the phase lag between two X-ray energy ranges for Fourier periods between 0.2 and 20 s. Taken together with our analysis of the power density spectrum, this indicates that shots tend to occur with two preferred durations and that the X-ray energy spectrum becomes harder as the shot progresses. This is consistent with an accretion model in which clumps of matter in the disk have two preferred sizes, and are heated by the release of gravitational energy as they drift from the outer to the inner region of the disk before disappearing into the black hole.

The X-ray flux from Cyg X-1 is variable on many timescales^{2,3}. Recently we showed⁴ that the time delay of the variations of high-energy X-rays from Cyg X-1 relative to those of the lower energy X-rays is inconsistent with the simple high-energy-gain inverse-Compton-scattering model of Cyg X-1 (refs 5, 6). Here we report analyses of further Cyg X-1 observations made with the Ginga satellite⁷.

Our data were obtained on 5–8 August 1987 with the large-area counters (LAC)⁸ onboard Ginga, when Cyg X-1 was in its low state ($\sim 11 \times 10^{-9}$ erg cm⁻² s⁻¹ in the range 2–20 keV). The observed energy ranges (and time resolutions) are 1.2–5.7 keV (1 ms), 5.7–24.4 keV (2 ms), 1.2–15.8 keV (1 ms), 15.8–24.4 keV (2 ms) in the PC mode⁸, and 1.2–37 keV in 12 channels with time resolutions of 8 ms in high bit rate and 63 ms in medium bit rate in the MPC-3 mode⁸.

In Fig. 1, some examples of the light curves of the observed X-rays from Cyg X-1 are shown. To examine the characteristics of these light curves, we calculated the complex Fourier com-

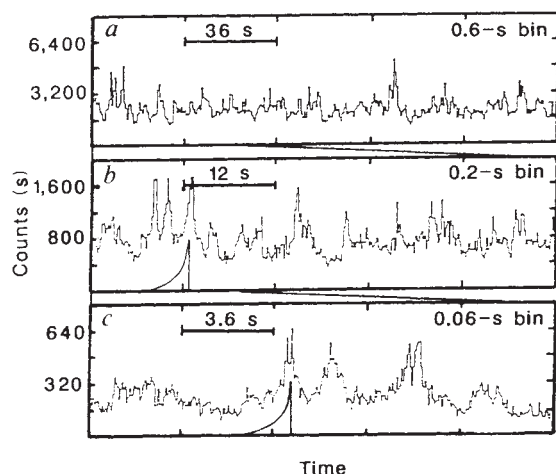


FIG. 1 Examples of the X-ray light curves of Cyg X-1 observed by Ginga. About one-third of the left part of the upper figure is shown in the lower figure. The shape of the X-ray shots given by equation (1) are also shown in b and c for decay time constants of 0.2 s and 0.7 s respectively.

ponents of the observed X-ray counts in the various energy ranges, and then calculated the power density spectra and the complex cross-spectra to get the phase lags of the time variations between the X-ray intensities of two different energy ranges at various Fourier periods. (The delay time at a Fourier period is given by (phase lag) $\times$ (Fourier period) $/ (2\pi)$.) The method is similar to the one described in refs 4 and 9. We also calculated the autocorrelation functions. We show some typical results in Figs 2 and 3.

We obtained the following results from our analyses: (1) The slope of the power density spectrum is zero at Fourier periods larger than ~ 10 s and increases gradually as the period decreases (Fig. 2). The slope is smaller for higher energy X-rays and for larger Fourier periods. These results are similar to the results obtained by Nolan *et al.*¹⁰ although their X-ray energy range was different (11–137 keV). The zero slope and their shoulder at ~ 6 -s period indicate that there are few shots of time duration larger than $\sim 6/(2\pi)$ s; (2) In the doubly logarithmic plots of phase lag against Fourier period (Fig. 3), there are two peaks: one is between periods of ~ 0.2 s and 3 s, and the other is between ~ 3 s and 20 s. We find that this type of double-peaked structure is independent of the X-ray energy (in the range 1–30 keV) and sampling time. The phase lags of Cyg X-1 in its low state obtained from 1983 data¹¹ (X-ray flux $\sim 8 \times 10^{-9}$ erg cm⁻² s⁻¹), seem to be consistent with our results but their uncertainties are larger. It seems, therefore, that this structure always exists in the low state of Cyg X-1. These results indicate that there may be two characteristic time constants in the time variation of the X-rays from Cyg X-1; (3) The phase lags θ depend on the ratio of the two X-ray energies, and is given by $\theta \approx A(p) \ln(E_2/E_1)$, where E_1 and E_2 are the X-ray energies, p is the Fourier period and $A(p)$ is the shape of the double-peaked function ($\theta \approx 0.2$ rad for $p = 0.8$ s). This indicates that the X-ray energy spectrum becomes harder as the X-ray shot progresses; (4) For Fourier periods < 0.2 s, we cannot find any pattern in the phase lag/period diagram. Here the phase lag has large uncertainties, which may be partly due to a change in their values with time and partly the result of large intrinsic uncertainties for the low coherence values (see ref. 12, for example) at these periods. For Fourier periods > 20 s, the phase lags have a large scatter (which depends on the Fourier periods and the observation times) in spite of the fact that the coherence is nearly 100% and so one might expect the uncertainty of the phase lag to be smaller than that for low coherence values; (5) The time duration of the autocorrelation function of lower energy X-rays is slightly larger than that of higher energy X-rays (by $\sim 20\%$, in the case of 1.2–5.7 keV and 15.8–24.4 keV). This fact together with the hard-X-ray lag indicates that the intensity of the hard X-rays in the shots should increase and should not decrease later than the soft X-rays.

To explain the phase-lag structure mentioned above, we investigated the following model. The X-rays from Cyg X-1 consist of many shots. In the shots, the energy spectrum becomes harder with increasing X-ray intensity. This increase of the X-ray intensity continues for some time and then the intensity decreases rapidly and almost simultaneously for X-rays of all energies. These characteristics are consistent with observations (3) and (5) above.

In the following, we examine the conditions necessary to explain the double-peak structure in the phase lag/period diagram. Consulting the autocorrelation functions, we model the double peak with two shots of different shapes, I_1 and I_2 which increase exponentially with different time constants,

$$\begin{aligned} I_{1l} &= 1.0 \exp(t/0.7) & I_{1h} &= 1.0 \exp(t/0.54) \\ I_{2l} &= 1.0 \exp(t/0.2) & I_{2h} &= 1.0 \exp(t/0.1) \end{aligned} \quad t \leq 0 \quad (1)$$

$$I_{1l} = I_{1h} = I_{2l} = I_{2h} = 0 \quad t > 0$$

where I_{1l} and I_{1h} ($i = 1, 2$) are the intensities of the low-energy and high-energy X-rays for the same shot. The time constants

are smaller for higher energy X-rays even in the same shot. The parameter t is $T - T_0$, where T is time and T_0 is the time at which the X-ray flux decreases suddenly. Although this sudden decrease may not be real, the almost simultaneous and rapid decrease in different X-ray energies is approximated by this simplification.

We assume that these two kinds of shots, I_1 and I_2 , occur independently and at random but with equal probabilities. This probability includes such cases as that in which the flux of one kind of shot is decreased by a certain factor and at the same time the probability of the occurrence of the same kind of shot is increased by the same factor, because these cases lead to the same results. Our model is different from Terrell's¹ because here the intensity gradually increases and the energy spectrum becomes harder as the shot progresses, and because two different kinds of the shots are assumed.

The simulated phase lags from two kinds of shots are shown by the dotted line in Fig. 3. Good agreement is obtained for the phase lag between Fourier periods 0.2 s and 20 s. Because the observed phase lag has a large uncertainty for the Fourier periods < 0.2 s, no firm conclusion can be drawn from them. With these two kinds of shots, we cannot get good agreement in the power density spectrum at the Fourier periods ≤ 0.6 s as shown by the dotted line in Fig. 2.

We therefore introduce two more rapidly changing shots, I_3 and I_4 . From autocorrelation functions their behaviours are

$$\begin{aligned} I_{3i} &= 2.0 \exp(t/0.05) & I_{3h} &= 2.0 \exp(t/0.04) \\ I_{4i} &= 1.0 \exp(t/0.01) & I_{4h} &= 6.0 \exp(t/0.004) \end{aligned} \quad t \leq 0.0 \quad (2)$$

$$I_{3L} = I_{3h} = I_{4i} = I_{4h} = 0.0 \quad t > 0.0$$

Thus we assume there are four different kinds of shots, which occur independently and at random with the same probability, in the sense mentioned above.

For this model we can get a similar power density spectrum as that obtained from the observations (Fig. 2) as well as the double-peaked phase-lag structure shown in Fig. 3. This result shows that the shots with time constants shorter than those given in equation (1) are necessary, although the time parameters of these added shots in equation (2) may not be unique and may change with time.

This multi-duration shot model in which the energy spectrum hardens as the shot progresses is consistent with the conclusion¹³ that a simple shot model with a fixed exponential time constant cannot simulate the observed autocorrelation function.

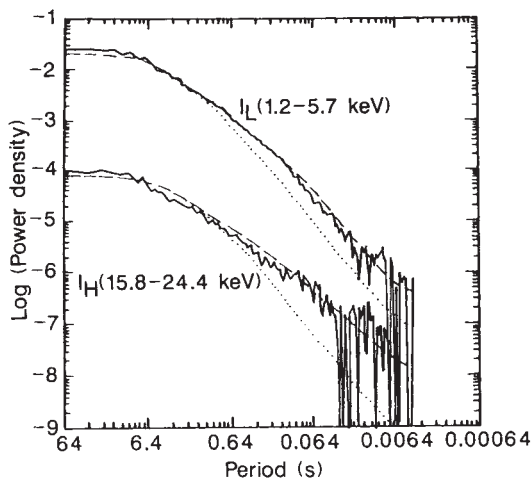


FIG. 2 Power density spectrum of the data taken on 5 August 13:26-18:31 UT in PC mode, and the power density spectrum calculated using equation (1) (dotted line) and equations (1) and (2) (dashed line).

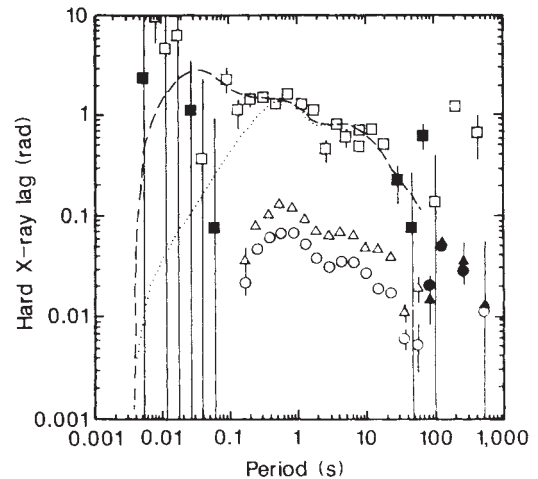


FIG. 3 The mean hard-X-ray phase lags for X-rays of different energies. The circles compare X-rays in the regions 1.2-4.7 keV and 4.7-9.3 keV. The triangles compare X-rays in the regions 1.2-4.7 keV and 9.3-14.0 keV. The closed circles and closed triangles indicate negative values, that is, the time variations of the soft X-rays lag those of the hard X-rays. These results are based on the observations of 6 August 11:49-8 August 12:04 UT in MPC-3 mode. We used all of the MPC-3 data to reduce the uncertainty. In this case the time resolution is 63 ms. The open and filled squares compare X-rays in the regions 1.2-5.7 keV and 15.8-24.4 keV. The filled square indicates a negative value. These results are based on the data taken on 5 August 13:26-18:31 UT in PC mode. The simulated phase lag obtained from equation (1) (dotted line), and equations (1) and (2) (dashed line) are also shown. The 5 August data and their simulated values have been multiplied by a factor of ten.

Although we use a sudden cut-off this model approximately simulates the general time behaviour of the X-ray shots from Cyg X-1 as shown in Fig. 1, in which we compare the shape of the shots given by the equation (1) with the observed X-ray light curves of Cyg X-1. Although the sudden decrease of the model shot is a simple approximation of the actual decrease of the shots, we can see that the shots of the time durations of about 0.2 s and 0.7 s are prominent X-ray shots observed on Cyg X-1.

Thus we have shown that the observed double-peaked phase-lag structure and the power density spectra can be explained by a model in which shots have two preferred durations, and in which the X-rays harden as the shot progress. We suggest that the release of gravitational energy as clumps of matter of two preferred sizes drift from the outer to the inner region of the disk before disappearing into the black hole could be the cause of the multi-duration shots in which the X-rays harden with time. These clumps of matter of two preferred sizes, possibly of two preferred masses, may be the result of physical conditions or dynamics of the inner part of the accretion disk around the black hole of Cyg X-1. $\square$

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Origin of 'normal-branch' quasiperiodic oscillations in low-mass X-ray binary systems

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QUASIPERIODIC intensity oscillations with frequencies in the range 6–10 Hz have recently been discovered in luminous low-mass X-ray binary systems^{1,2}. These so-called 'normal-branch' oscillations (NBOs) seem to be a nearly universal feature of those systems with luminosities near the Eddington critical luminosity L_E (refs 3, 4), at which the outward force of radiation balances gravity. Here we report the results of numerical simulations of the approximately radial accretion flow expected near the neutron-star component, which show that the flow becomes overstable (that is, the flow oscillates, with increasing magnitude) when the luminosity rises to within a few per cent of L_E . If the radial inflow begins ~ 300 km from the neutron star (as expected), the frequency of the oscillations is ~ 5 –10 Hz, comparable to the observed frequencies of NBOs. The time variation in optical depth is substantial, whereas the variation in luminosity is relatively small. The predicted amplitudes and phases of the resulting intensity oscillations agree with observations⁵ of the NBOs. We suggest that the 10–20 Hz oscillations in intensity observed in Scorpius X-1 when it is on the flaring branch^{3,4} are caused by photohydrodynamic modes that are excited by the oscillations in the radial flow and grow when the luminosity exceeds L_E .

The quasiperiodic oscillations recently discovered in the luminous low-mass X-ray binaries seem to be of two types, the so-called horizontal-branch oscillations (HBOs), which are strongest when a source is in the horizontal-branch spectral state and have frequencies in the range 20–50 Hz, and the NBOs, which appear when a source is in the normal-branch spectral state^{3,4,6}. At least one source, Cyg X-2, sometimes shows both types of oscillations simultaneously⁷. The magnetospheric beat-frequency model^{8–10} is widely considered the most promising explanation of the HBOs, but there is as yet no generally accepted explanation of the NBOs⁶.

Here we summarize an investigation of the NBO mechanism suggested as part of a recently proposed^{11,12} unified physical model of accretion flows and X-ray emission in low-mass X-ray binaries. In this model, gas supplied by the binary companion star accretes onto a weakly magnetic neutron star. Most of the gas approaches the neutron star through a keplerian accretion disk, which is disrupted by the magnetic field of the neutron star at two or three stellar radii. The pressure of radiation escaping from the disk drives some of the gas into a corona above the inner disk. Radiation drag causes gas in the inner corona to lose its angular and vertical momentum and to fall approximately radially toward the neutron star. The resulting mass flux represents ~ 10 –20% of the total mass flux onto the star. The radial mass flux is proportional to the luminosity, and hence is approximately constant for luminosities near L_E . The radial flow ends in a compact central corona surrounding the small magnetosphere of the neutron star.

The structure of the radial flow is extremely sensitive to small variations in the mass flux or luminosity, when the luminosity is close to the Eddington luminosity, $L_E \equiv 4\pi GMm_p c / \sigma_T$. Here M is the mass of the neutron star, m_p is the proton mass, c is the speed of light and σ_T is the cross-section for Thomson scattering. This extreme sensitivity, and the unavoidable time lag between changes in the inward mass flux from the corona and the resulting luminosity changes, indicated that the radial flow would oscillate for luminosities sufficiently close to L_E (refs

11, 12). Simple estimates of the expected frequency of the oscillation and the variation with photon energy of its amplitude and phase agreed qualitatively with the observed properties⁵ of the NBOs.

We have simulated the radial inflow of gas captured from the inner disk corona using an explicit one-dimensional time-dependent lagrangian radiation hydrodynamics program with artificial viscosity. Previous studies of time-dependent radial accretion by neutron stars^{13,14} have demonstrated the important influence of radiation drag on the structure of the flow. However, these studies considered only very dense, supercritical ($L \gg L_E$) flows, assumed that all of the mass flux was in the radial flow, and followed the time development only for very short times (≤ 0.16 s in the case of ref. 13 and ≤ 0.03 s in the case of ref. 14). Hence, these studies are not directly relevant to an assessment of the NBO mechanism considered here. By contrast, our radiation hydrodynamics program allowed us to explore the effects of both disk and radial mass fluxes and to follow near-critical radial flows for many inflow times.

In our program, the gas flow is calculated from the gas flow and radiation field at the previous time step. The radiation field is then calculated by solving the time-independent transfer equation to order v/c . This approach is valid because radiation escapes through the flow in $\leq 10^{-2}$ inflow times. The luminosity is the sum of the luminosity produced by the disk flow (assumed constant) and the luminosity produced by the radial flow. The luminosity produced by the radial flow consists of (1) the luminosity of the compact central corona, including the kinetic and thermal energy of each mass shell entering the corona and the gravitational potential-energy difference between the outer radius of the corona at 20 km and the neutron star radius R at 10 km, and (2) the work done on the radiation from the central corona by the converging radial flow. As a result, the luminosity is a function of radius. The radial flow was modelled by some 400–800 mass shells. Among other tests, the program successfully reproduced analytic solutions representing free-fall, inflow in the presence of a near-critical radiation flux¹⁵, and hydrostatic equilibrium. A typical simulation, lasting 10–20 inflow times, required two hours of CPU (central processing unit) time on a Cray X-MP/48.

The behaviour of the inflow was found to depend on the assumed mass flux $\dot{M}_d$ onto the star from the disk flow and the

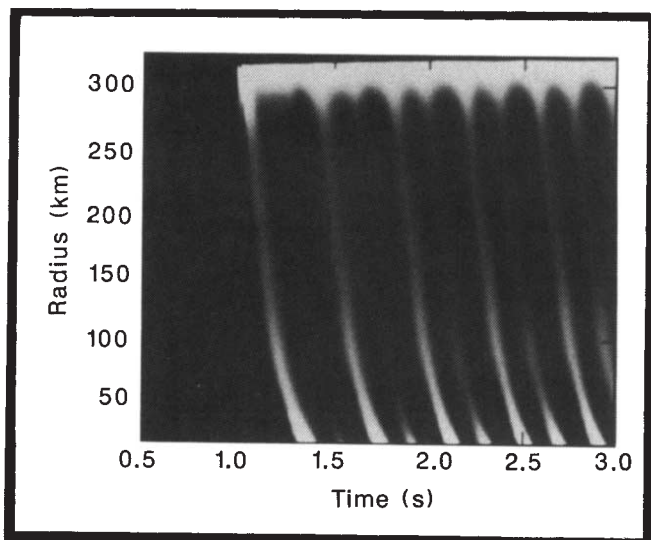


FIG. 1 Grayscale plot showing the gas density in a typical simulation relative to the gas density in the corresponding unperturbed flow, as a function of radius and time. Higher density regions are lighter; lower density regions, darker.

mass flux $\dot{M}_r$ onto the star from the radial flow. We specify these by the dimensionless parameters $\mu_d \equiv \dot{M}_d/\dot{M}_E$ and $\mu_r \equiv \dot{M}_r/\dot{M}_E$, where $\dot{M}_E$ is the mass flux that produces an accretion luminosity L_E . The relative importance of the radiation force is described by the dimensionless parameter $\varepsilon \equiv 1 - (L_\infty/L_E) = 1 - \mu_d - \mu_r$, the relative difference between the luminosity measured in a fixed frame far from the star L_∞ and the Eddington luminosity. Only two of the three parameters μ_r , μ_d and ε are independent; here we use μ_r and ε . When there is an adequate supply of matter, we expect $L_\infty \approx L_E$, and hence $\varepsilon \ll 1$.

We were able to simulate steady, near-critical inflows for a large grid of μ_r and ε values. In these simulations, shells of constant mass were added to the outside of the flow ($r \approx 200$ – 300 km) at a steady rate to simulate the approximately constant inward mass flux from the corona produced by radiation drag. In different simulations, shells were added with inward velocities ranging from one-half the speed of sound to ten times the speed of sound. The qualitative structure, temporal behaviour and stability properties of the radial flow were found to be insensitive to the velocity of the outermost shells.

Analytical solutions¹⁵ as well as our numerical simulations (B.F., F.K.L. and G.S.M., manuscript in preparation) show that the structure of steady near-critical radial flows is governed by competition between gravitational and radiation-pressure forces. The flows discussed here are supersonic except near the compact central corona and, when the mass shells added to the outside edge of the flow are given subsonic inward velocities, close to the outer edge of the flow. Thus, gas-pressure forces have only a modest effect. The outer part of the flow is optically thin to scattering. Radiation drag becomes important at or inside the outer critical radius $r_2 \equiv GM/\varepsilon c^2$. Gas captured from the inner disk corona accelerates inward until its radial velocity reaches the value $\varepsilon c/2$ at which the co-moving luminosity equals the critical luminosity L_E and the acceleration vanishes. Inside the inner critical radius $r_1 \equiv (\mu_r/\varepsilon)R$ the flow becomes optically thick, radiation trapping is small but significant, and the flow velocity decreases linearly with radius until gas pressure gradients become important¹⁵. The time t_f required for gas captured from the inner disk corona to reach the neutron star is dominated by the inflow time from the outer part of the radial flow and hence is $\approx r_c/v_r(r_c) \approx 2r_c/\varepsilon c$, where $r_c \sim 300$ km is the characteristic radius at which approximately radial inflow begins.

We studied the stability of near-critical flows by setting up a steady flow and then disturbing the luminosity of the central corona or the inward mass flux from the outer boundary. The response of steady flows to perturbations of the luminosity or the mass flux was very similar. Figures 1 and 2 show the results of a typical simulation in which regular oscillations developed. For this run, $\mu_r = 0.05$ and $\varepsilon = 0.01$. The flow included ~ 600 shells and the inflow time from the outer edge was ~ 0.3 s. Shells were added to the outer edge of the flow with an inward velocity equal to the sound speed. The simulation was followed for ~ 10 inflow times. From $t = 0$ – 0.6 s, an interval corresponding to two inflow times, mass shells were added to the outer edge of the flow at a constant rate and the flow was allowed to relax to a steady-state solution by adjusting the luminosity at the inner boundary so that the luminosity at infinity remained constant. Beginning at 0.6 s, the luminosity at the inner boundary was calculated self-consistently from the radial inflow.

At 1.0 s, the flow was disturbed by adding 30 extra mass shells (equivalent to 5% of the mass in the radial flow) to the outer edge of the flow; after this time, mass shells were again added to the outer edge at a constant rate. The leftmost light striation in Fig. 1 shows the progress of the density enhancement as it falls from the outer edge of the flow at ~ 300 km toward the compact central corona at 20 km. One inflow time later, at 1.3 s, the density perturbation reached the inner boundary and created a brief, small increase in the luminosity of the central corona. This luminosity perturbation caused a small amount of gas to

accumulate in the outer part of the flow and near 150 km, where the inflow velocity is most sensitive to the luminosity. When these density maxima subsequently reached the inner boundary, they created primary and secondary luminosity peaks, causing the cycle to repeat. The secondary peak grew to about the same size as the primary peak within a few cycles, and the flow subsequently oscillated with frequency $f_{\text{osc}} \approx 2/t_f \approx 6$ Hz. All the simulations that displayed regular oscillations had an oscillation frequency $\approx 2/t_f$.

Our simulations show that perturbations in the mass flux or luminosity are damped for $\mu_r/\varepsilon \lesssim 4$ but grow exponentially for $\mu_r/\varepsilon \gtrsim 4$. For $\mu_r/\varepsilon \approx 4$, disturbances of the mass flux or luminosity produce regular oscillations. These stability properties can be understood simply as follows. Any density perturbation $\Delta\rho_i$ at the outer edge of the radial flow at time t_i is advected toward the neutron star, producing a change in the mass flux at the inner boundary of relative size $\Delta\rho_i/\rho_i$ one inflow time later, at time t_{i+1} . The resulting relative change in the luminosity is $\Delta\varepsilon_{i+1} \propto -\mu_r\Delta\rho_i/\rho_i$. This change is communicated to the outer boundary on the (very short) radiation diffusion timescale. Because the inward mass flux at the outer boundary is constant, the density of the flow there depends only on the local velocity, which we have shown above varies with luminosity as ε . Thus, the change in luminosity produces a change in the density at the outer boundary at time t_{i+1} of size $\Delta\rho_{i+1}/\rho_{i+1} \propto (\mu_r/\varepsilon)\Delta\rho_i/\rho_i$, and hence the amplitude of the oscillation grows exponentially at a rate $\gamma \sim \ln(\mu_r/\varepsilon)/t_f$. The actual boundary between stability and instability ($\mu_r/\varepsilon \approx 4$) is determined by the competition between this growth mechanism and damping effects, such as Compton cooling and spreading of the density perturbations caused by gas pressure gradients and radiation drag. These latter effects are also the reason that even a sharp disturbance in the mass flux or luminosity produces a relatively sinusoidal response.

For radial flows beginning at ~ 200 – 300 km, the simulations that exhibited regular oscillations all had oscillation frequencies $f_{\text{osc}} \sim 5$ – 10 Hz. These frequencies are comparable to the observed frequencies of NBOs at their onset near the middle of the normal branch^{3,4,7}. The electron-scattering optical depths τ_{es} of the inflows that exhibited regular oscillations were all ~ 10 . The relative amplitude $\Delta L/L$ of the luminosity oscillations tends to be much smaller than the relative amplitude $\Delta\tau_{\text{es}}/\tau_{\text{es}}$ of the oscillations in the electron-scattering optical depth. This is to be expected, because the luminosity cannot vary by an amount greater than $\sim \varepsilon L$ without disrupting the flow, whereas variations in the luminosity of this magnitude produce large variations in

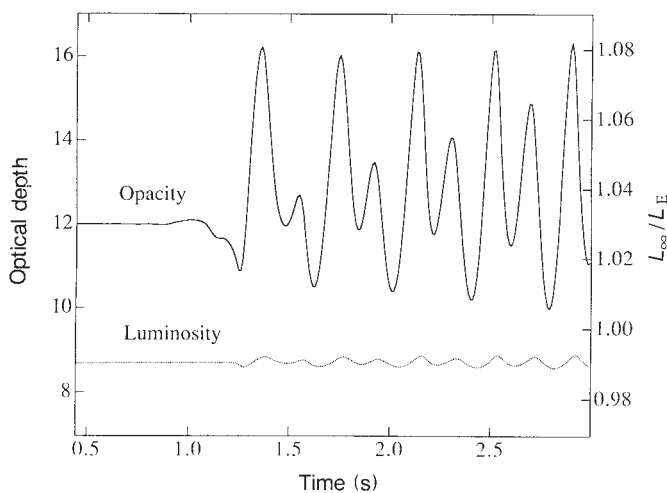


FIG. 2 Variation of the electron-scattering optical depth (left scale) and the total luminosity (right scale) during the simulation of Fig. 1. Note the quiescence of the flow before it is disturbed at 1.0 s. The optical depth varies by $\sim 30\%$ whereas the luminosity varies by $<1\%$.

the density and optical depth of the flow. Thus, our simulations tend to support the earlier suggestion^{11,12} that NBOs are primarily oscillations in optical depth rather than luminosity. Numerical calculations of the effect of optical-depth oscillations on the X-ray spectrum (G.S.M. and F.K.L., manuscript in preparation) show that such oscillations cause the spectrum to rotate about a pivot energy that depends on the spectrum but is in the range 2–7 keV for observed spectra. Thus, our mechanism predicts that the oscillations above and below the pivot energy will be $\sim 180^\circ$ out of phase and that the oscillation amplitude will go through a minimum near the pivot energy. These results agree quantitatively with the observed properties⁵ of NBOs.

The frequency of the oscillations is governed by the outer radius of the radial-flow region and the inflow velocity there. Both are functions of ε . For a given mass flux in the radial flow, oscillations begin at a definite value of ε . The mass flux in the radial flow is determined by the luminosity of the star and the structure of the inner disk corona far inside the outer radius of the disk, but still far outside the neutron-star magnetosphere. Therefore, the radial mass flux, the value of ε at which oscillations develop, and the frequency of these oscillations at their onset are likely to be similar in all the luminous low-mass X-ray binaries. If the radial flow begins ~ 300 km from the neutron star (as expected), the frequency of the NBOs will be ~ 6 Hz at onset. The increase in the NBO frequency from ~ 6 to ~ 10 Hz which is observed as sources approach the bottom of the normal branch may be caused by shrinking of the radial-flow region as the luminosity approaches L_E and mass loss from the inner disk increases¹¹.

The radial-flow oscillations discussed here are likely to excite photohydrodynamic (PHD) modes that have similar frequencies¹¹. The amplitude of these modes is expected to increase as the luminosity approaches and then exceeds L_E (ref. 16). The resulting segregation of the accreting gas from the outflowing radiation allows the neutron star to continue to accrete gas even when the luminosity exceeds L_E . It also increases the effective gravity seen by the accreting gas, causing the frequency of the PHD modes to increase. This would explain why the ~ 10 -Hz oscillations seen in Sco X-1 at the bottom of the normal branch go over smoothly to ~ 10 -Hz oscillations at the bottom of the supercritical flaring branch, and why the oscillation frequency then increases^{3,4,7} as Sco X-1 moves up the flaring branch. Segregation of the accreting gas from the outflowing radiation also reduces the average optical depth seen by the radiation, which would explain the X-ray spectral changes that occur as Sco X-1 moves up the flaring branch¹¹. Finally, at sufficiently high luminosities we expect a broad spectrum of PHD modes to be excited, which would account for the observed^{3,4} widening of the oscillation peak into a broad spectrum of noise as Sco X-1 moves further up the flaring branch. $\square$

New features in Saturn's atmosphere revealed by high-resolution thermal infrared images

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FIFTEEN years ago, it was found¹ that Saturn has a very different thermal infrared (7–13 μm) brightness distribution from that observed at visible wavelengths. Single-detector scans of Saturn at 11.7 μm (ref. 1) showed emission increasing strongly towards the south pole, which was attributed in part to 12- μm ethane (C_2H_6) emission in the upper atmosphere of Saturn, enhanced by an atmospheric temperature inversion. Subsequently, it was confirmed that the observed brightening in ethane was concentrated towards the pole, and was not simply a general limb-brightening effect². A similar brightening has been seen in the vibrational ν_4 band of methane near 7.8 μm ³ and in the hydrogen (3–1) pressure induced rotational line near 17.0 μm . Here we present observations of the stratospheric infrared emission structure on Saturn made using a 58×62 pixel imaging array camera system at 7.8, 11.6 and 12.4 μm . The high-spatial-resolution (1.3-arcsec full width at half maximum) global images show a variety of new features including a narrow equatorial belt of enhanced emission at 7.8 μm , a prominent symmetrical north polar hotspot at all three wavelengths, and a mid-latitude structure which is asymmetrically brightened at the east limb. These results confirm the polar brightening and reversal in position predicted by recent models^{4,5} for seasonal thermal variations of Saturn's stratosphere.

The first⁴ seasonal model for the ground-based observations of Saturn's stratosphere predicted that the maximum southern polar temperature (1-mbar level) occurs near the vernal equinox (1980), whereas the maximum insolation occurs at southern solstice (1973). Infrared spectra acquired in 1980 and 1981 by the Voyager 1 and 2 Infrared Interferometric Spectrometer (IRIS) instruments showed strong south polar brightening in the 1,304 cm^{-1} methane (CH_4) and 821 cm^{-1} ethane (C_2H_6) bands, and the data were used to derive the atmospheric temperature profile from the troposphere to the stratosphere^{6,7}. The spectra required differing tropospheric temperature profiles (50–300 mbar range), confirming the presence of significant thermal asymmetry between the northern and southern hemispheres. Voyager 1 IRIS found that the north polar region was ~ 20 K colder at 1 mbar than the equator at that epoch. Improved seasonal climatic models were developed for the troposphere⁸ and stratosphere⁵ of Saturn. Since the saturnian vernal equinox in 1980, the orientation of Saturn has changed to near northern solstice, so that the southern hemisphere is now both in the shadow of the ring system and obscured from view by the rings. The model of Bezaud and Gautier⁵ predicts enhanced temperatures at the north pole during the present epoch. Caldwell *et al.*⁹ reported warming of the northern regions in the constant sunlight, based on raster scanning observations in 1986. No new infrared maps of Saturn have been published since the Voyager encounter, although a programme of synoptic infrared mapping is underway (J. Caldwell and G. Orton, personal communication). The subsequent development of large-format thermal infrared array detectors has made global imaging of the planets possible. Here we report the results of such observations of Saturn in the wavelength range 7–13 μm .

The array camera system uses a 58×62 pixel gallium-doped-silicon 5–17 μm direct-readout photoconductor-array detector

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manufactured by Hughes (Santa Barbara Research Center). The array and optics are cooled to 10 K with liquid helium to optimize detector performance and minimize instrumental thermal background. A single off-axis parabolic mirror (3.0-inch focal length) re-images the telescope focal plane onto the array detector with a plate scale of $0.26 \text{ arcsec pixel}^{-1}$. The parabolic mirror also re-images the telescope's $f/35$ secondary mirror at a cold aperture stop for off-axis background suppression. The system has a set of fixed interference filters (7.8, 8.7, 9.8, 10.3, 11.6 and $12.4 \mu\text{m}$) with $\Delta\lambda/\lambda \approx 0.1$, and 5–14 μm circular variable-filter wheel with $\Delta\lambda/\lambda = 0.04$. (See ref. 10 for a detailed description of the array camera system.)

The 7.8-, 11.6- and $12.4\text{-}\mu\text{m}$ images of Saturn were acquired at the NASA Infrared Telescope Facility (IRTF) at Mauna Kea on the mornings of 26 and 28 March 1989 under seeing-limited observing conditions that resulted in 1.3-arcsec-resolution images (full width at half maximum, FWHM). The diffraction limit of the 3-m IRTF telescope is 1.04 arcsec (FWHM) at $12.4 \mu\text{m}$. The field of view of the 58×62 array was $14.9 \times 16.3 \text{ arcsec}$ in right ascension and declination respectively, permitting the whole disk of Saturn to be imaged in a single frame. The array camera system was background limited on the IRTF telescope with a measured observational noise-equivalent flux density of $1\sigma = 0.05 \text{ Jy min}^{-1/2} \text{ pixel}^{-1}$ ($1 \text{ Jy} = 10^{-26} \text{ W m}^{-2} \text{ Hz}^{-1}$).

Figure 1 compares our three filter spectral bands with spectra of Saturn acquired by Voyager IRIS at mid-latitudes and at the south pole^{6,7}. The $11.6\text{-}\mu\text{m}$ and $12.4\text{-}\mu\text{m}$ bands are dominated by C_2H_6 emission from Saturn's stratosphere. Our spectral synthesis models (not shown) reproduce the Voyager IRIS spectra and demonstrate that the fraction of the intensity in our images at the pole contributed by C_2H_6 would be ~ 0.9 at $11.6 \mu\text{m}$ and $12.4 \mu\text{m}$. At the equator these fractions would be ~ 0.5 and ~ 0.7 respectively. The $7.8\text{-}\mu\text{m}$ pass-band is sensitive primarily to CH_4 , with negligible continuum contribution. Terrestrial tropospheric CH_4 absorption has an important role at $7.8 \mu\text{m}$ because the ν_4 lines of this gas are strong absorbers. Although the terrestrial lines are pressure broadened ($\sim 0.1 \text{ cm}^{-1}$ (FWHM), or $\sim 22 \text{ km sec}^{-1}$), their obscuring effect was minimized in these observations by the Doppler shifting of the saturnian CH_4 lines that results from Saturn's large geocentric velocity (-28 km sec^{-1}).

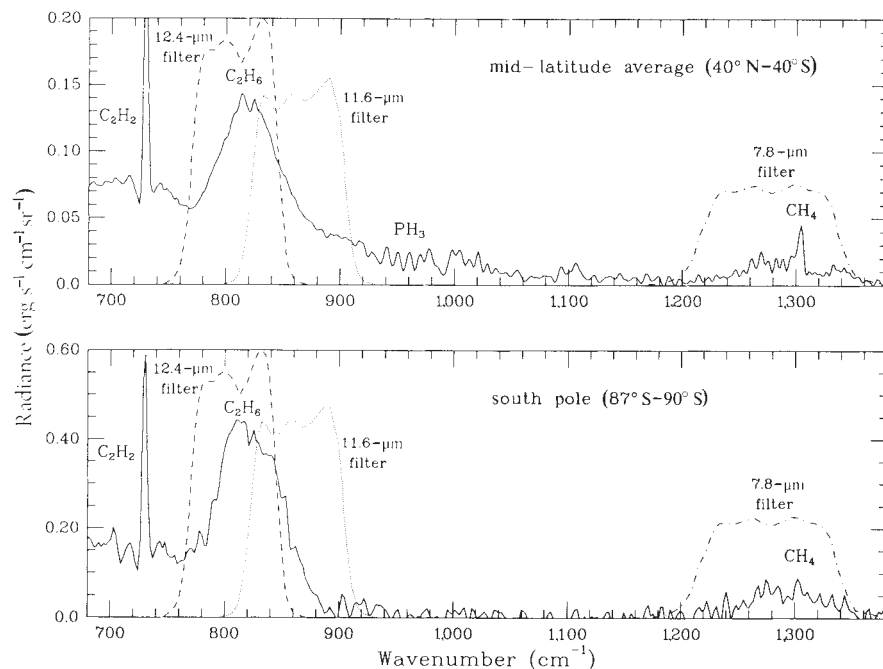
The photometric imaging results are shown in Fig. 2 in grey-scale and linear contour-plot forms. The aspect geometry of Saturn and its rings is presented for reference. Saturn's axis of rotation was inclined 24.7° toward the Earth on 28 March 1989. Figure 3 shows the north-south intensity profiles generated by averaging the intensity in the projection of a $5^\circ \times 5^\circ$ area on the surface of Saturn, along the longitude line at 60° E of the central meridian (normalized to the polar maximum). This longitude strip was chosen because of its high-contrast $7.8\text{-}\mu\text{m}$ intensity structure, clearly showing the equatorial belt.

The observed peak brightnesses at the north pole are 2.7, 9.7 and $20 \text{ Jy arcsec}^{-2}$ at 7.8, 11.6 and $12.4 \mu\text{m}$ (using the star Sco α as a calibrator), corresponding to brightness temperatures of 137, 113 and 115 K. The brightness of the polar hotspot increases continuously from about 60° N latitude to the pole, is symmetrical in longitude, and is centred at the rotational pole (to within $\pm 5^\circ$). This is opposite to the data taken by Voyager 1 IRIS near the saturnian vernal equinox, when the north pole was found to be 20 K colder than the equator at the 1-mbar level. Our observations thus confirm the seasonal reversal in the equator-to-pole temperature gradient predicted by the models^{4,5}.

A narrow equatorial belt of emission is clearly evident in the $7.8\text{-}\mu\text{m}$ image of Saturn (Fig. 2b). This new emission feature was not seen by Voyager IRIS. The belt is $\sim 15^\circ$ wide and is $\sim 10\text{--}30\%$ brighter than the adjacent mid-latitude region (Figs 2, 3). Corresponding, but weaker, equatorial emission is seen at $12.4 \mu\text{m}$ (C_2H_6), but is not detected unambiguously at $11.6 \mu\text{m}$. In the $7.8\text{-}\mu\text{m}$ image obtained on 28 March 1989 at 10:30 UT (Fig. 2b) there is also an irregular, clumpy structure extending northward from the belt to a latitude of about $+30^\circ$ near the meridian. This feature is not present in the $7.8\text{-}\mu\text{m}$ image of 26 March when Saturn was presenting the opposite side to Earth, indicating that the structure rotates with the planet. An isolated bright spot is seen on the equatorial belt at $7.8 \mu\text{m}$ near the east limb on 28 March, which is also not apparent on 26 March.

The images show prominent mid-latitude structure. The region in latitudes $30^\circ\text{--}40^\circ \text{ N}$ is bright at all three wavelengths and is flanked by a darker region at lower latitudes, and by a prominent dark circumpolar 'collar' near 50° N latitude. The $30^\circ\text{--}40^\circ \text{ N}$ bright region shows a large-scale longitudinal asymmetry, with the east limb being brighter. We estimated that Saturn's east

FIG. 1 Infrared spectra of Saturn obtained by averaging Voyager 1 IRIS observations at Saturn's vernal equinox in November 1980. Relative filter transmittances for the array-camera spectral bands at 7.8, 11.6 and $12.4 \mu\text{m}$ are shown for comparison (not indicative of absolute sensitivity). The contributions of CH_4 , C_2H_6 , C_2H_2 and the underlying H_2 continuum in each filter band can be seen. The south pole spectrum shows enhanced stratospheric emission in the hydrocarbons because of increased temperature and higher emission angle (note differing radiance scales).



limb should have been $\sim 10\%$ brighter than the west limb through our $7.8\text{-}\mu\text{m}$ filter. The Doppler shift of the approaching (east) limb was -37.2 km sec^{-1} compared with -20.0 km sec^{-1} for the west limb at the time of our observations. The transmission of the Earth's atmosphere in the wings of CH_4 absorption lines was therefore higher for Saturn's east limb. This does not, however, explain the asymmetry at 11.6 or $12.4\text{ }\mu\text{m}$ because C_2H_6 absorption is negligible in the Earth's atmosphere. This

asymmetry is believed to be real, and may be similar to the large-scale longitudinal brightness-temperature structure seen on Jupiter¹¹.

Saturn's rings were not detected in emission. The ring system is cold but opaque to $7\text{--}13\text{ }\mu\text{m}$ radiation. The outer C Ring is warmest at $85 \pm 1\text{ K}$ and exhibits optical depth of $\tau = 0.10 \pm 0.03$ at normal incidence⁷; at the present inclination $\tau \approx 0.24$, and the B Ring would have $\tau \approx 1.94$. The sharp southern edge of the

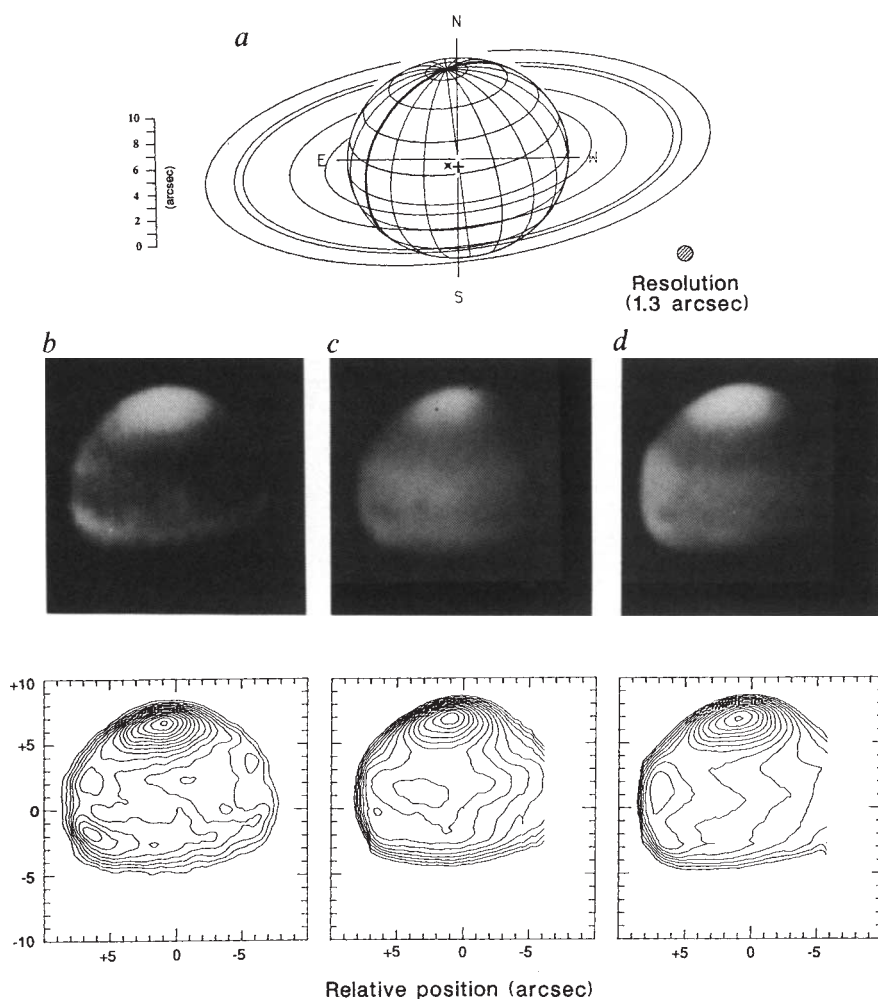
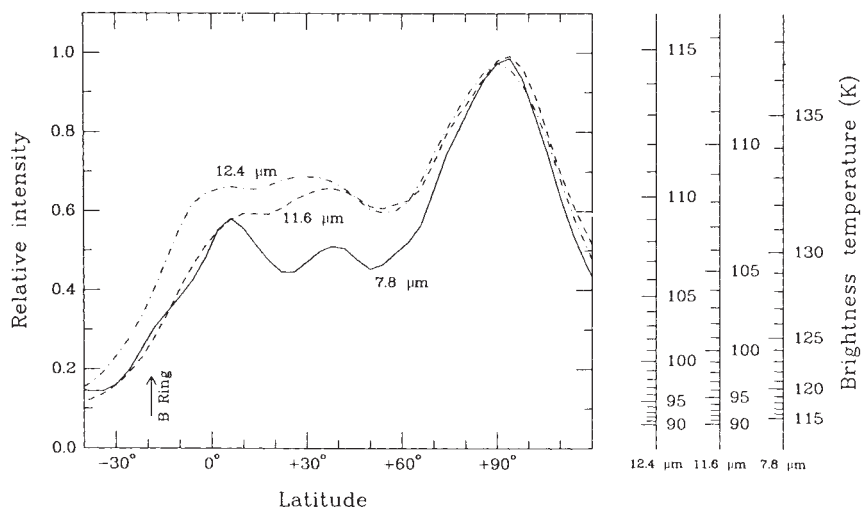


FIG. 2 *a*, Orientation of Saturn for the 28 March observations; the cross-hairs indicate the disk centre and coordinate geometry on the sky, the 'x' is the sub-solar point, and the '+' is the sub-Earth point. The longitude line at 60°E indicates the intensity profile data in Fig. 3. *b*, $7.8\text{-}\mu\text{m}$ image of the disk of Saturn (smoothed in a 3×3 pixel synthetic aperture, 1 pixel = 0.26 arcsec) and corresponding linear intensity contour plot (contour interval = 0.1 , peak brightness = $2.7\text{ Jy arcsec}^{-2}$) with position offsets in right ascension and declination from the disk centre indicated in arcseconds. Saturn's ring system is not detected. *c*, Same for $11.6\text{ }\mu\text{m}$ (peak = $9.7\text{ Jy arcsec}^{-2}$); the dark spots near the east limb result from localized array defects not affecting the rest of the image. *d*, Same for $12.4\text{ }\mu\text{m}$ (peak = 20 Jy arcsec^{-2}).

FIG. 3 Relative Saturn intensity profiles at 7.8 , 11.6 and $12.4\text{ }\mu\text{m}$, extending from the projected inner edge of the B Ring at the south, over the north pole to the north-west limb. The observed intensity was averaged in a projected $5^\circ \times 5^\circ$ area on Saturn, along a constant longitude line 60°E of the central meridian (see Fig. 2*a*) and normalized to unity at the polar maximum in each filter band. This longitude strip was chosen for high surface contrast. Absolute intensities are indicated by the brightness-temperature scales. The narrow equatorial belt is most prominent at $7.8\text{ }\mu\text{m}$ ($\sim 30\%$ contrast). The strong polar emission rises continuously at latitudes greater than about 60°N at all three wavelengths.



images is defined by the inner edge of the ring system and/or the effects of the ring shadow, and this is shown clearly in Fig. 2. The 7.8- μm equatorial belt is parallel to the occulting inner edge of the ring system, although uncertainties of up to 3° in tilt and 5° in displacement with respect to the rotational equator cannot be excluded because of latitude structure in the belt. The position of the equatorial emission belt is therefore not inconsistent with the latitude of the magnetic-dip equator¹² at 6°N . The Earth exhibits thermospheric emission in the form of two bright belts of 130-nm atomic-oxygen emission near the magnetic Equator, associated with electron-density enhancements (the Appleton anomalies)¹³. The Earth also has a strong electric current (the equatorial electrojet) flowing east-west along the magnetic Equator and only a few degrees of latitude in width¹⁴. A similar current system may exist on Saturn, and electron collisional excitation could contribute to the 7.8- μm methane vibrational emission observed. Other effects associated with Saturn's rings could be significant, including convective transport of atmospheric gas to high altitudes along a sharp temperature discontinuity at the inner edge of the ring shadow. Such convective transport could lead to enhanced stratospheric temperatures by ortho-para H_2 conversion¹⁵. More exotic processes, such as deposition of material from the rings, cannot be excluded and will be considered as part of a subsequent program of more detailed thermal infrared observations. $\square$

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Self-organized criticality in the 'Game of Life'

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THE 'Game of Life'^{1,2} is a cellular automaton, that is, a lattice system in which the state of each lattice point is determined by local rules. It simulates, by means of a simple algorithm, the dynamical evolution of a society of living organisms. Despite its simplicity, the complex dynamics of the game are poorly understood. Previous interest in 'Life' has focused on the generation of complexity in local configurations; indeed, the system has been suggested to mimic aspects of the emergence of complexity in nature^{1,2}. Here we adopt a different approach, by using concepts of statistical mechanics to study the system's long-time and large-scale behaviour. We show that local configurations in the 'Game of Life' self-organize into a critical state. Such self-organized criticality provides a general mechanism for the emergence of scale-free structures³⁻⁵, with possible applications to earthquakes^{6,7}, cosmology⁸, turbulence⁹, biology and economics¹⁰. By contrast to these previous studies, where a local quantity was conserved, 'Life' has no local conservation laws and therefore

represents a new type of universality class for self-organized criticality. This refutes speculations that self-organized criticality is a consequence of local conservation¹¹, and supports its relevance to the natural phenomena above, as these do not involve any locally conserved quantities. The scaling is universal in the sense that the exponents that characterize correlation functions do not depend on details of the local rules.

The grand and general question is how the laws of physics—which describe processes on the microscopic scale—can lead to a world organized on all scales. The idea of 'self-organized' is that it is in the nature of nonlinear processes to organize mathematical systems into structures that have order on all length scales. If this tendency is generally present in such mathematical systems, then we would also expect the natural world to contain structures on all scales. Here we demonstrate this by choosing 'at random' an entirely local algorithm, the 'Game of Life', and showing that the model evolves to a stationary state where small perturbations create objects of all sizes.

The canonical example of self-organized criticality is a 'pile of sand'. Imagine building the pile by slowly adding particles. As the pile grows, there will be bigger and bigger avalanches. Eventually a statistically stationary state is reached in which avalanches of all sizes occur, that is, the correlation length is infinite. Thus, in analogy with equilibrium thermodynamical systems the state is 'critical'. It is also self-organized because no fine-tuning of external fields was needed to take the system to the critical state: the criticality is unavoidable. This is in contrast to the behaviour for an equilibrium phase transition,

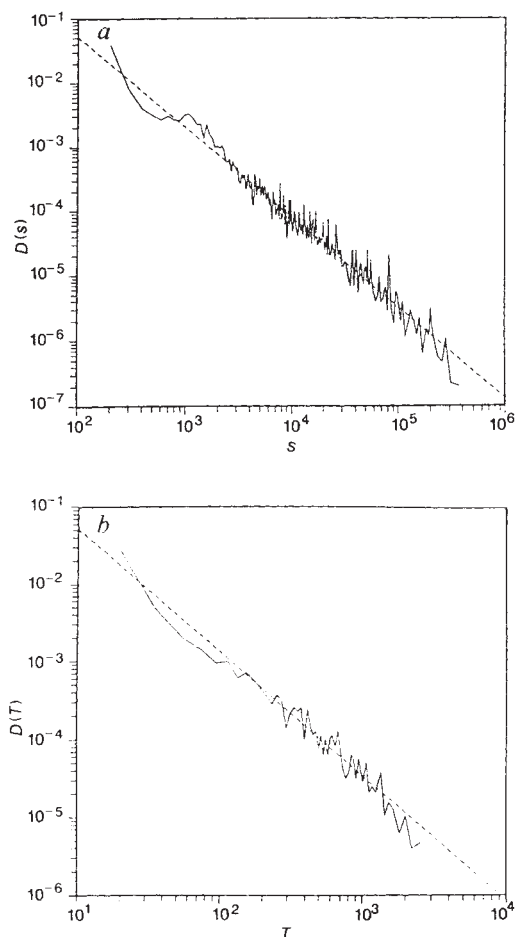


FIG. 1 a, Log-log plot of distribution of cluster size for a 100×100 system. b, Distribution of the duration for evolution of clusters. The deviation from power-law behaviour for large clusters is a finite-size effect.

in which one has to tune, for instance, the temperature to arrive at the critical point.

The 'Game of Life' is defined on a square lattice. There are two states on each lattice site, representing the presence or absence of a live individual. The rules for the evolution of 'Life' are very simple. (1) The fate of a live individual depends on its eight nearest neighbours; it will die at the next time step if there are less than two (over-exposure) or more than three (over-crowding) live neighbours; otherwise it will remain alive. (2) At a dead site, a new individual will be born at the next time step only if there are exactly three live neighbours. Figure 2 includes some configurations of still life, cyclic life and propagating gliders. Numerous local stationary configurations can be generated by these simple rules, representing variety and complexity of local stable societies. We will focus, however, on the collective behaviour of live organisms.

We simulate 'Life' on finite lattices of sizes up to 150×150 . Open (absorbing) boundary conditions are generally chosen, but the scaling region of the spatial and temporal correlations is not affected by boundary conditions. Of course, the finite size of the system cuts off very-large-scale features. We studied the following process. Starting with a random distribution of live sites, the system evolves according to rules (1) and (2) until it comes to 'rest' in a simple periodic state with a distribution of local still life and simple cyclic life (with our method of generation, cyclic structures of long period are extremely rare and essentially never encountered). The system is then perturbed on a randomly chosen local site, by adding a live individual, and is then allowed to evolve according to the rules until it comes to rest again. As the process is repeated, the system evolves into a statistically stationary state, which does not depend on the initial configuration. The properties of this state are the object of our investigation.

First, we measure the total activity s , defined as the total number of births and deaths following a single perturbation. Figure 1a shows the distribution of 'clusters' of size s averaged over 40,000 perturbations. The distribution seems to be a power law, $D(s) \propto s^{-\tau}$, $\tau \approx 1.4$. Figure 1b shows the distribution of durations of perturbations $D(T)$. This also seems to be a power law, $D(T) \propto T^{-b}$ with $b \approx 1.6$. The fact that the activity does

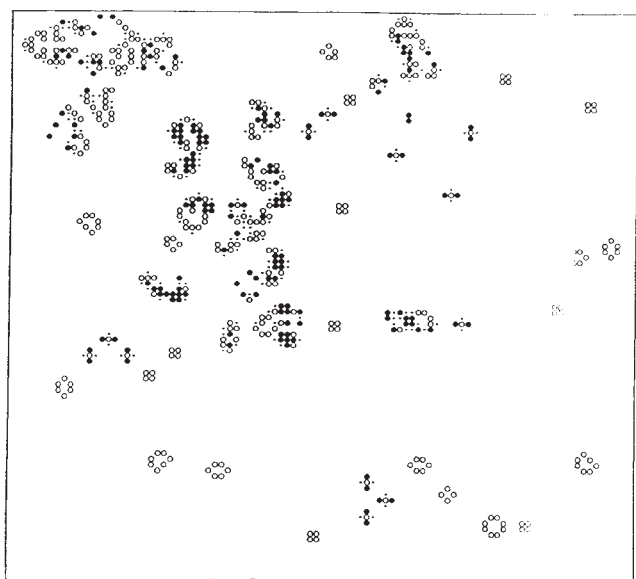


FIG. 2 Configuration of a 100×100 system responding to a perturbation. Live passive sites are open circles, dying active sites are filled circles; the dots indicate sites where birth will take place at the next time step. Note configurations of still life (clusters of open circles), cyclic life with period 2, and propagating gliders.

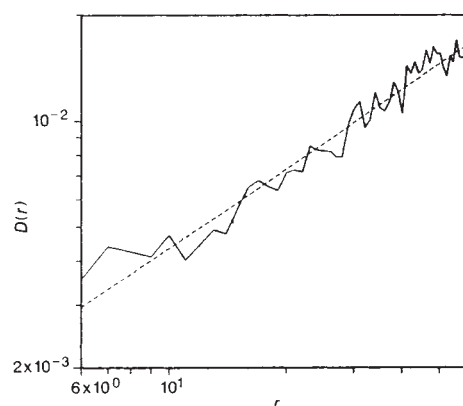


FIG. 3 Number distribution of active sites at a distance r from a given active site near the centre of a 150×150 system.

not decay or explode exponentially (become chaotic) indicates that life and death are highly correlated in time and space: the system has evolved into a critical state. Figure 2 shows a configuration of the activity in the middle of a perturbation. Notice the clustering of the activity, which indicates that 'Life' is sustained on a fractal. The number distribution of active sites at a distance r from a given active site in an active cluster increases with r as $D(r) \propto r^{D-1}$, where the fractal dimension $D \approx 1.7$ (Fig. 3). The fractal structure of the activity is not apparent in a single configuration of our rather small system, but becomes clear when averaged over many configurations. These power laws indicate that the stationary state of 'Life' is critical, with avalanches through the system on all scales. In biology, these avalanches may be thought of as the response to slow changes in the environment.

We have shown numerically that the 'Game of Life' operates at a self-organized critical state, characterized by critical exponents τ , b , D and others. By analogy with traditional critical phenomena, we do not expect the critical properties to depend on the particular features of the rule, and thus they are universal. Also the model could be 'accidentally' critical, in the sense that modifications drive it away from criticality (or the model could be operating close to, but not at, the critical point so that deviations from power laws were not detected in the calculation). To check this, we have constructed a three-state model, which does not exhibit complex local configurations, but nevertheless seems to be critical, with exponents which seem to be the same as those of the 'Game of Life'⁸. The essential feature of the simplified model is the existence of propagating particles acting as messengers communicating between different parts of space. The simplified model is defined in any number of dimensions, and thus may be more tractable to standard renormalization-group methods known from critical phenomena. Further studies of this model may help to elucidate the critical properties of 'Life'.

The 'Game of Life' is not meant to be a realistic model for any particular system, but serves to demonstrate how large-scale structures can arise in complicated extended dynamical systems. Nevertheless, it has been suggested from studies on much more complicated mathematical models of co-evolution (S. Kauffman, personal communication) that biology indeed operates at a self-organized critical state like the one described here. $\square$

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Entropy of amorphous ice

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THE configurational entropies of amorphous solids reflect certain aspects of their structures, in particular the numbers of accessible molecular configurations. The configurational entropy of vitreous silica has been estimated theoretically¹ as no more than $5.8 \text{ J K}^{-1} \text{ mol}^{-1}$, which agrees reasonably with experiment. That of low-density amorphous ice has been estimated theoretically² as $\sim 6.3 \text{ J K}^{-1} \text{ mol}^{-1}$ more than that of ice Ih, using a model that assumes a continuous random network of hydrogen bonds. This value corresponds to ~ 2.1 more configurations per molecule than in ice Ih, and seems consistent with the result for vitreous silica¹. In principle, these entropies can be obtained from the change of heat capacity from the glass to the liquid and the entropy of freezing of the liquid at equilibrium, but such measurements have not proved possible because the liquid generally crystallizes too quickly. Here we show that the entropies can be estimated approximately by another method: from the thermodynamics of the transformations of, for example, both ice Ih³ and low-density amorphous ice⁴ to high-density amorphous ice. The entropy of high-density amorphous ice relative to that of ice Ih is estimated as $2 \pm 1 \text{ J K}^{-1} \text{ mol}^{-1}$, and that of high-density relative to low-density amorphous ice is estimated as $1 \pm 0.5 \text{ J K}^{-1} \text{ mol}^{-1}$. The entropy of low-density amorphous ice relative to ice Ih is therefore $\sim 1 \text{ J K}^{-1} \text{ mol}^{-1}$. The earlier estimates^{2,5} of this quantity are therefore several times too high. These low values limit the amount of disorder that can be present in the amorphous phases.

When ice Ih is compressed to $\sim 10 \text{ kbar}$ at 77 K (ref. 3) it transforms to a high-density amorphous ice, several properties of which have been measured^{3,4,6–8}. Low-density amorphous ice transforms to a high-density form in a similar way⁴, but at less than half the pressure at which ice Ih transforms. Both of these amorphous phases can be recovered at 77 K and ambient pressure, apparently without any significant change except for a uniform and reversible expansion^{3,4}.

The pressure p_e at which the two phases are in equilibrium with one another is described by the equation

$$p_e = -\frac{\Delta A_e}{\Delta V_e} = -\frac{\Delta U_e}{\Delta V_e} + T_e \left(\frac{\partial p}{\partial T} \right)_e \quad (1)$$

$$\equiv p_e(1) + p_e(2), \quad (2)$$

where ΔA_e , ΔV_e and ΔU_e are the difference between the Helmholtz functions, the volumes, and the internal energies of the two phases at equilibrium, respectively, T_e is the temperature of the equilibrium, $(\partial p / \partial T)_e$ is the slope of the equilibrium line for the two phases, and $p_e(1) \equiv -\Delta U_e / \Delta V_e$ and $p_e(2) \equiv T_e(\partial p / \partial T)_e$. The difference between the enthalpies of the two phases at low pressure is the difference in the internal energy, and the internal energy changes little with pressure because it depends only on the thermal expansion and the compression. Therefore, the difference between the internal energies of the two phases under pressure can be represented, to a reasonable approximation, by the difference in enthalpy at zero pressure.

The difference between the enthalpies of high-density amorphous ice and ice Ih is $-2,125 \text{ J mol}^{-1}$ at $\sim 100 \text{ K}$ (ref. 6). As

the change of volume at the transformation of ice Ih to high-density amorphous ice is $-4.2 \pm 0.1 \text{ cm}^3 \text{ mol}^{-1}$ (ref. 3), then, for the equilibrium between the two phases

$$p_e(1) = 5.1 \text{ kbar} \quad (3)$$

if the effect of temperature on the enthalpy changes can be neglected in a first approximation.

The contribution of the term $T_e(\partial p / \partial T)_e$ to equation (1) is not known by direct experiment, but its range can be estimated by plotting the equilibrium pressures of ice Ih and liquid water in the range $253\text{--}273 \text{ K}$ (ref. 9) (see curve 1, Fig. 1) and extrapolating them to low temperature. Four extrapolations have been made, assuming that the entropy change at the transformation of ice Ih to high-density amorphous ice is either 1, 2, 3 or $6 \text{ J K}^{-1} \text{ mol}^{-1}$ and that the temperature of the glass transition at these pressures is independent of the pressure and is 150 K . This temperature is 25 K above the value measured by Klug and Handa¹⁰ for low-density amorphous ice, and is the temperature at which high-density amorphous ice crystallizes when it is warmed in the range $10\text{--}30 \text{ kbar}$ (ref. 11). The values of $p_e(2)$ were then calculated from the temperature and the assumed slopes, and the quantities p_e , from equation (2), are plotted as the circles at 100 K in Fig. 1. The slopes of the equilibrium lines were calculated from the known volume changes at the transformations and the assumed entropy changes, and the calculated equilibrium lines are plotted in Fig. 1. The melting points at high temperatures and pressures were extrapolated in three ways to meet the slopes of the equilibrium lines for which the entropy changes at the line are 1, 2, 3 and $6 \text{ J K}^{-1} \text{ mol}^{-1}$. Three lines extrapolate reasonably well, and they show that the entropy of

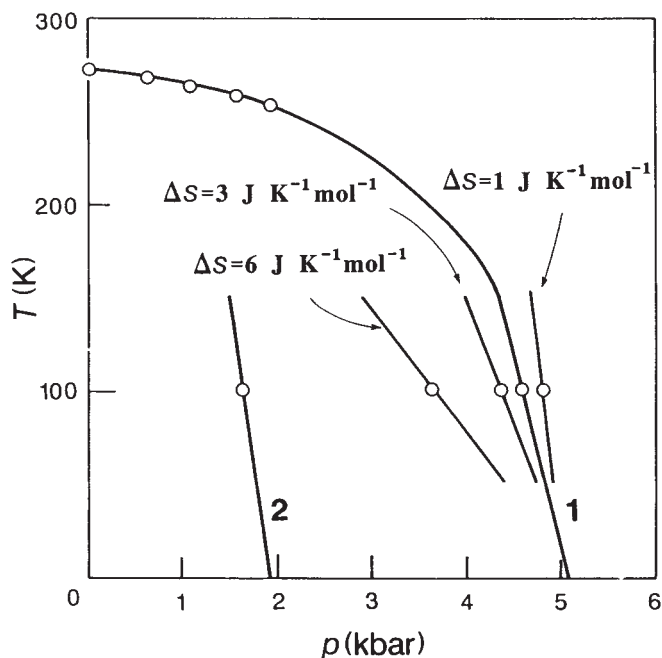


FIG. 1 Curve 1 is the equilibrium line between ice Ih and the liquid plotted in the range $0\text{--}2 \text{ kbar}$ (ref. 9) and extrapolated to zero temperature as described in the text. Curve 2, below $\sim 150 \text{ K}$, is the equilibrium line between low-density and high-density amorphous ice, as described in the text. Curve 2 does not exist above $\sim 150 \text{ K}$ because the two amorphous forms of ice melt; it is drawn only to suggest that a continuity of states might exist at low temperatures, if measurements could be made quickly enough. The equilibrium lines that would occur below the glass transition if the entropy changes at the transformation of ice Ih to high-density amorphous ice were 6, 3 and $1 \text{ J K}^{-1} \text{ mol}^{-1}$, are drawn through the equilibrium pressures p_e predicted by equation (1).

the transformation is $2 \pm 1 \text{ J K}^{-1} \text{ mol}^{-1}$. The entropy change of $6 \text{ J K}^{-1} \text{ mol}^{-1}$ seems to have little relation to the extrapolated melting curve, and so an entropy change of this magnitude is much too great to be consistent with the phase diagram. The entropy of a proposed 'continuous random network' model of $\sim 6.3 \text{ J K}^{-1} \text{ mol}^{-1}$ (ref. 2) seems to be about three times too high, and so is also an alternative estimate⁵.

The equilibrium between low-density and high-density amorphous ice can be discussed in a similar way. The change of enthalpy at the transformation of high-density amorphous ice to low-density amorphous ice at low pressure⁶ is -224 J mol^{-1} in the range 85–105 K, and -531 J mol^{-1} in the range 105–114 K, the sum being -755 J mol^{-1} . As the volume change at the transformation is $-4.0 \pm 0.3 \text{ cm}^3 \text{ mol}^{-1}$ (ref. 4), the contribution of the term $-\Delta U_e/\Delta V_e$ to the equilibrium pressure between the two phases is 1.9 kbar. The equilibrium between ice Ih and low-density amorphous ice cannot, of course, be followed above the glass transition at $\sim 150 \text{ K}$. Nevertheless, the equilibrium line exists below the glass transition, because the system is metastable, not unstable, and it seems unlikely to be greatly different from curve 2 of Fig. 1. The configurational entropy of low-density amorphous ice must be less than that of high-density amorphous ice and greater than that of ice I so the slope of curve 2 is greater than that of curve 1. The slope of curve 2 below 100 K is -2.6 bar K^{-1} , and, from this and the volume change reported above, the entropy change is $\sim 1.0 \text{ J K}^{-1} \text{ mol}^{-1}$, with an estimated uncertainty of $\sim 0.5 \text{ J K}^{-1} \text{ mol}^{-1}$.

As the difference between the entropies of ice Ih and high-density amorphous ice is $\sim 2 \text{ J K}^{-1} \text{ mol}^{-1}$ and the difference between the entropies of low-density and high-density amorphous ice is $\sim 1 \text{ J K}^{-1} \text{ mol}^{-1}$, the difference between the entropies of ice Ih and low-density amorphous ice is $\sim 1 \text{ J K}^{-1} \text{ mol}^{-1}$. The difference in entropy cannot be less than this value, and is surely less than the entropy difference between ice Ih and high-density amorphous ice. In both low-density and high-density amorphous ice, the hydrogen bonding, as measured by the shape of the uncoupled Raman band of isolated hydrogen-bonded O—H—O or O—D—O groups⁷, is essentially complete, and no unbroken hydrogen bonds could be detected. This greatly limits the amount of disorder that can be present in the two phases. This is not inconsistent with random-network models and indicates that the principal difference between the crystalline and the amorphous phases and between the two amorphous phases is a relatively small degree of disorder.

The difference in the amount of disorder can be described by the difference in the number of configurations per molecule at the transformations. It is ~ 1.27 for the transformation of ice Ih to high-density amorphous ice, ~ 1.13 for the transformation of high-density amorphous ice to low-density amorphous ice, and ~ 1.13 for the transformation of ice Ih to low-density amorphous ice.

Our method may be applied to many substances whose melting line declines with increasing pressure, such as ammonium fluoride I (ref. 12), ammonium fluoride monohydrate, many structure II clathrate hydrates such as tetrahydrofuran clathrate hydrate, indium antimonide, germanium and many other materials³. □

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Optical absorption microscopy and spectroscopy with nanometre resolution

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SEVERAL variants of the scanning tunnelling microscope (STM) have been developed since its invention¹. Here we present the first demonstration of a new type of microscope, based on the STM, that is capable of recording optical absorption spectra with nanometre (that is, molecular) resolution. Our starting point is conventional photothermal spectroscopy, in which the temperature rise owing to the absorption of tunable monochromatic light is measured, giving a direct measurement of the absorption spectrum of a sample as a function of illuminating wavelength. The spatial resolution of such measurements is determined by the sizes of the pump beam or detector, whichever is smaller. We use the STM to measure the 'thermoelectric' tunnelling voltage due to absorption. Conventional photothermal measurements^{2,3} have been limited to a resolution of $\sim 1 \mu\text{m}$, although we have recently developed a thermal sensor with a spatial resolution of $\sim 50 \text{ nm}$ (ref. 4), and suggested its use for spectroscopy on that scale⁵. The STM, however, permits resolution of about 1 nm, and as the image is controlled by the thermal properties of the sample, our technique can image variations in the thickness of surface films, and changes in sub-surface characteristics, as well as chemical variations.

The range of other microscopes using the same concept of scanning and controlling a sensor of small dimensions over a surface⁶ as is used in the STM now enable measurement of temperature⁷, optical properties^{8,9}, magnetism¹⁰, electrical potential¹¹, dielectric properties¹², ionic conductance¹³ and surface forces^{14–16} to be made with extremely high spatial resolution. Effort has also been directed towards the identification of chemical species in a spatially localized region. Methods used include phonon-assisted tunnelling spectroscopy¹⁷, room-temperature tunnelling spectroscopy^{18,19} and inverse

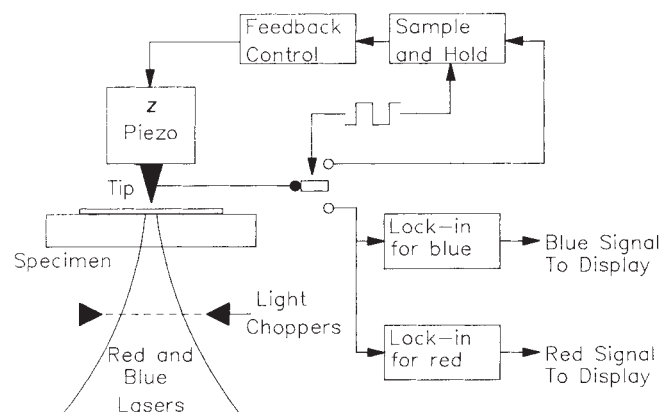


FIG. 1 Schematic of absorption microscope. Beams from a 'reference' He-Ne laser at 633 nm and from a tunable argon-ion laser are independently chopped at frequencies of 1 kHz and 1.5 kHz respectively. The beams are then combined and focused to a $2\text{-}\mu\text{m}$ spot on the sample directly below the tip of the STM. With the tunnelling feedback loop closed, the gap resistance is regulated to $500 \text{ k}\Omega$ and surface expansion measured. With the z-piezo position held constant the junction is isolated and the a.c. 'thermoelectric' voltage developed across the gap is synchronously detected at 1.0 and 1.5 kHz.

photoemission²⁰. So far, however, it is unclear whether any of these techniques will be able to characterize complex macromolecules.

Our microscope is based on a 'tunnelling thermocouple' which, in principle, can measure 'temperature' down to the atomic scale. We illuminate the junction region of an STM with a chopped laser tuned to a wavelength of spectroscopic interest, resulting in periodic heating of the tunnel junction. Because the

tip and sample are made of dissimilar conductors, a potential difference is developed across an external circuit. Variations in the amount of light absorbed by the specimen will lead to variations in the temperature of the junction and hence to variations in the voltage. Thus the thermoelectric voltage is directly related to the optical absorption of the specimen in the junction region. By scanning the tunnelling tip over the specimen we therefore obtain a map of optical absorption with the spatial resolution of an STM. The absorption of the tip is constant as a function of position and approximately constant as a function of wavelength; the temperature of the tip is therefore controlled by the temperature of its immediate environment and so the image contrast variations reflect changes in thermoelectric voltage that are due to changes in the absorption of the specimen.

The thermoelectric voltage can be understood in the following way. As the tip and sample electrodes come within tunnelling range ($\leq 5 \text{ \AA}$) of each other, their electronic states become strongly coupled and their Fermi levels tend to equalize because of the two-way electron tunnelling across the gap. Under conditions of local thermodynamic equilibrium, and in the case where the spatial extent of the thermal variations in the sample are large as compared with the electron scattering lengths, this voltage becomes the classical thermoelectric voltage between tip and sample electrode and the system acts as a regular thermocouple junction with the junction resistance replaced by the tunnelling resistance. The thermoelectric coefficient, however, may be different when the tip and sample are not in thermal equilibrium; calculations for this case have been performed in another context²¹. In our microscope, using a tungsten tip and gold specimen, the a.c. voltage developed across the tunnel junctions was found to be the same, within the experimental uncertainty, as that developed when the tip was deliberately driven into the gold film. The thermal state of the tip is therefore unchanged by contact. The measured thermoelectric coefficient of $3 \mu\text{V K}^{-1}$ is in good agreement with the published²² value for a gold-tungsten junction, implying that the tip and specimen are at approximately the same temperature.

The schematic of our system is shown in Fig. 1. A conventional STM is used to maintain a constant distance between tip and sample. From the *z*-piezo voltage we obtain a measure of the topography of the specimen and of the local thermal expansion. To measure temperature, the tunnelling feedback loop is repetitively disabled and all external bias removed from the tip; the open-circuit potential developed across the gap then forms a measure of the local thermoelectric potential and hence of the extent to which the pump beams have been absorbed. To eliminate sensitivity to small changes in tip-sample separation²³, we measure the thermoelectric voltage with a high impedance across the tunnel junction. The need for high-impedance measurement of voltage led us to use a novel feedback mechanism in which the junction was biased using a constant current.

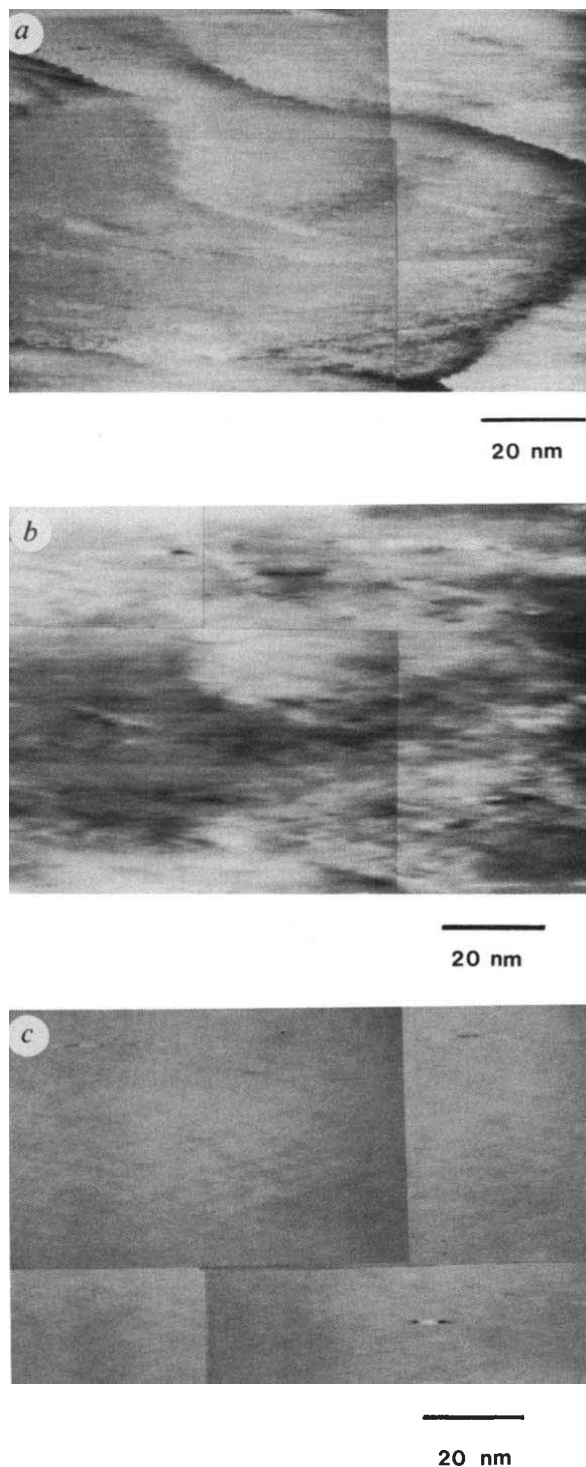


FIG. 2 *a*, Topographic view of 20-nm annealed gold film on mica. *b*, 'Thermal' image corresponding to illumination at 632.8 nm (reference image). *c*, 'Thermal' image at 476.5 nm divided by reference image; note lack of local spectroscopic contrast. Scan rate 7 s per line.

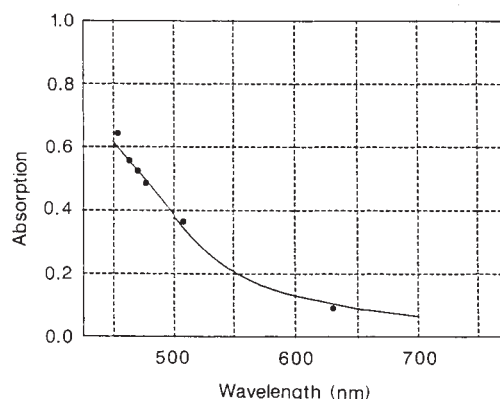


FIG. 3 Calculated optical absorption spectrum of 20-nm gold film (solid curve) compared with experimental data (dots).

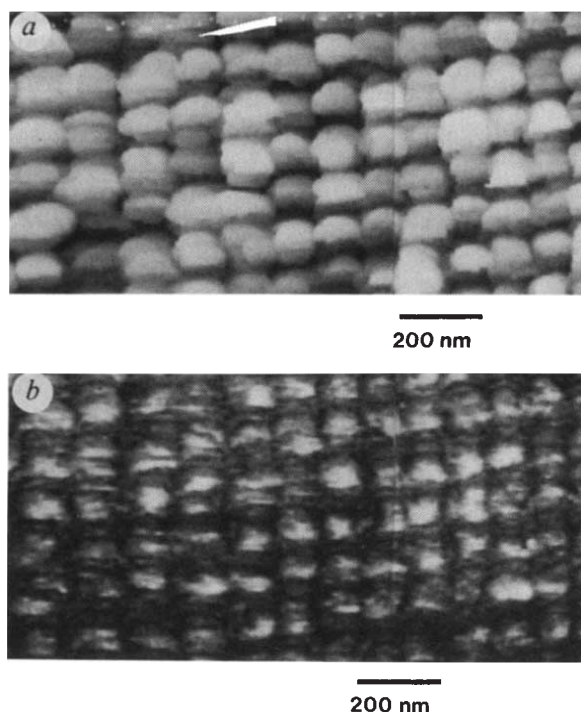


FIG. 4 *a*, Topographic scan over a sample consisting of 300-Å-diameter aluminium dots of 300-Å thickness deposited on a fused-quartz substrate with a 1,000-Å pitch and overcoated with 500 Å of gold. *b*, corresponding 'thermal' image at 514 nm; scan rate 30 s per line.

Measurement of the potential across the gap in the presence of bias gives a measure of gap resistance; in the absence of bias it gives a measure of thermoelectric voltage. Two illuminating beams are normally used. A tunable argon-ion signal-beam output at 1 kHz which gives a spectroscopic measure of absorption as a function of wavelength is normalized by a He-Ne reference-beam output at 1.5 kHz to eliminate any systematic variations in sensitivity arising from changes in the character of the tunnel junction—for example, variations in the chemical composition or thermal conductivity of the sample—as the tip is scanned across the sample. The ratio of signal to reference beam will remain unchanged by such variations as long as the optical absorption of the sample is unchanged.

Four separate images are recorded simultaneously; the tunnelling image, the He-Ne reference thermal image, the signal thermal image and the surface-expansion image (that is, the amplitude of the z -variation of tip position with time at the chopping frequency). Figure 2*a* shows an image of an annealed 20-nm gold film on mica using the tunnelling signal. Atomic steps are clearly resolved; the finest features in the image show a resolution of ~ 1 nm, substantially better than the thermal-diffusion length in the sample. This is a consequence of the near-field measurement of temperature, which varies inversely with the lateral distance from the absorber and so falls off rapidly outside of the beam. Figure 2*b* shows the corresponding thermal reference image taken at a wavelength of 632 nm. The same features are clearly resolved indicating a comparable resolution to the tunnelling image. We see, however, features in the thermal image that are not present in the tunnelling image and vice versa. This is because the tunnelling image in this case is only sensitive to variations in surface topography whereas the thermal image is sensitive to both variations in optical absorption and thermal properties of the sample-substrate system. Thus, differences in the mica topography that are filled in by the annealed gold film will not appear as a modulation in the tunnelling topography image but are present in the thermal image because of thickness variations in the gold resulting in corresponding variations in the optical absorption. Also, any

variations in the sub-surface adhesion of the gold film onto the substrate will appear as a modulation in the thermal image but will not be recorded in the tunnelling image. The normalized thermal image in Fig. 2*c* shows little or no variation as expected. This is because the amount of light absorbed is approximately proportional to film thickness for thin films; the ratio of red to blue absorption is therefore independent of film thickness, to first order. The ratio of the signal to reference image is therefore constant. The surface-expansion images (not shown) recorded in these series of experiments showed structure, but with a spatial resolution that was about two orders of magnitude worse. This is expected—if we assume a typical expansion coefficient for solids of 10^{-5} K^{-1} and a sensitivity for z displacement in the tunnelling microscope of 10^{-2} Å , the smallest absorption feature that could be resolved would be 1,000 Å for a 1° modulation in temperature.

To demonstrate the spectroscopy capability of our system, we stopped the x - y scanning motion across the gold film and plotted the normalized thermal signal as a function of pump optical wavelength by rapidly scanning the wavelength. As shown in Fig. 3, these results are in good agreement with the absorption spectrum of gold calculated from published²² values of the optical parameters.

Figure 4 shows a scan over an overcoated array of 30-nm aluminium dots. The thermal absorption image clearly shows the period of the underlying structure and seems to show detail not seen in the tunnelling image. We believe that this may represent real sub-surface structure not seen in the topographic scan.

The sensitivity of our microscope is limited, at present, by Johnson noise in the tunnel gap. With a thermocouple sensitivity of $3 \mu\text{V K}^{-1}$ and a gap resistance of 100 k Ω this limitation corresponds to a minimum-detectable temperature difference of $1.4 \times 10^{-2} \text{ K}$. If power P is absorbed by a thin sample of diameter D on top of a substrate with thermal conductivity κ , we can write an expression for the temperature change $\delta\theta$ (ref. 24) $\delta\theta = 2P/(\pi\kappa D)$. If we assume a 1-mW laser focused to a 2- μm spot and a 1-nm-diameter absorber on mica (close to our experimental conditions) we obtain $\delta\theta = 0.24 \text{ K}$ which is easily detectable with our microscope.

As our technique has the resolution to record optical absorption spectra of single molecules, we believe that with further refinement it may be useful in biology and medicine to distinguish between different molecular species—such as antigens on the surfaces of cells. The chemical identification of the bases by spectroscopy coupled with nanometre-scale microscopy should make this a candidate technique for sequencing DNA.

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Defect-induced stabilization of diamond films

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THE growth of diamond thin films by low-pressure vapour deposition should find technological applications in such diverse fields as hard coatings for cutting tools, lens coatings, heat sinks and electronics¹⁻⁴. Under such growth conditions diamond should be unstable relative to graphite, yet diamond is formed in practice. Present approaches to describing the growth of diamond explain this paradox by means of a variety of surface kinetic reactions that are controlled by concentrations of adsorbed hydrogen and hydrocarbon radicals⁵⁻⁸. Here we propose a simpler explanation, that high vacancy concentrations are present near the growth face of the diamond film. The formation energy of vacancies in diamond is lower than in graphite, so that large vacancy concentrations (1-8%) can raise the formation energy of graphite above diamond, permitting nucleation and stable growth of diamond. This quasi-thermodynamic model is fundamentally different from the kinetic models for diamond film growth, and predicts that the growth of films with low defect concentrations will be difficult.

The growth of diamond films at low pressure¹⁻⁴ has been interpreted strictly as a kinetic non-equilibrium process because of the known thermodynamic stability of graphite under these conditions. Theoretical ideas describing the kinetic growth have focused on the specific plasma species (such as neutral hydrogen) and kinetic processes responsible for accelerating the growth of the cubic material over graphite⁵⁻⁸. By considering the concentrations of hydrogen and methane, reactions are selected that are able to describe the experimentally measured concentration dependence of the growth rates⁵. Other models invoke the concept of hydrogen stabilization of the (111) surface up to temperatures of 1,000 °C. This, they claim, accounts for the decrease in the efficiency of diamond growth at higher temperatures⁷. The kinetic approaches proposed so far may help explain why diamond grows but they cannot account for the nucleation of diamond on non-diamond surfaces.

In a quasi-thermodynamic picture⁹ we compare the binding energy of diamond and graphite and include the energy of a large concentration of vacancies. The vacancies change the relative binding energies so that diamond becomes more stable than graphite. In this picture the low-pressure growth conditions lead to a large vacancy concentration near the growth face where layers are incompletely grown. Vacancies at the growth face affect the probability of sticking and orderly growth for each material—graphite and diamond. Kinetic processes with the assistance of thermal energy or bombardment energy, which enable the atoms to attach or be detached from the surface, will result in the more tightly bound materials growing more rapidly. Normally this would be expected to be graphite because of its greater binding energy, but the presence of vacancies changes the balance of energy. Thus, the dominant mechanism for stabilizing the growth of diamond is the effective free-energy lowering of the diamond film relative to the graphite film by incorporation of vacancies.

We approximate the quasi-equilibrium free energy for diamond and graphite with a concentration n of vacancies as

$$F = F_b + nF_d \quad (1)$$

where F_b is the binding energy of the materials with negligible defect concentrations and F_d is the free energy of formation of a defect, or vacancy. A transition to diamond will occur if the

formation energy of vacancies is smaller in diamond and

$$n > \frac{F_b(\text{diamond}) - F_b(\text{graphite})}{rF_d(\text{graphite}) - F_d(\text{diamond})} \quad (2)$$

where r is the ratio of vacancies in graphite and diamond.

Thus, a significant energy difference in the energies of defect formation may change the balance between the close binding energies of graphite and diamond. In insulating materials such as diamond the Fermi energy strongly affects the formation energy of charged defects:

$$F_d(q, \mu) = F_d(q, \mu_0) + q(\mu - \mu_0) \quad (3)$$

where q is the charge of the defect and μ , μ_0 are the Fermi energy and reference Fermi energy respectively^{10,11}. The second term arises from the energy of the electron added to or taken from the electron reservoir with an energy μ .

The growth conditions for diamond films as practiced today induce Fermi energy shifts significantly away from mid-gap at the growth surface. Both in the plasma- and filament-assisted chemical vapour deposition (CVD) processes commonly used for the growth of diamond films, there is significant electron bombardment of the growing surface; adding electrons is equivalent to raising the Fermi energy. Evidence for charging of the surface has been found (T.D.M., manuscript in preparation).

Recent calculations of the vacancy formation energy in diamond¹² with the Fermi energy at mid-gap and in graphite¹³ give energies of 7.2 eV and 7.6 eV respectively, and the difference in cohesive energies of diamond and graphite is ~0.03 eV. Using this result, assuming $r \approx 1$, 8% vacancies at the growth face would stabilize diamond with respect to graphite. For the vacancy in diamond, however, shifting the Fermi energy is very effective at further lowering the defect energy because many charge states of the vacancy are possible¹². As the Fermi energy is varied in diamond the formation energy of the diamond vacancy can be lowered by as much as 4 eV (negative defects by raising the Fermi energy and positive defects by lowering the Fermi energy). There is no analogous change in the vacancy formation energy in graphite because it is metallic. Thus, $F_d(\text{graphite}) - F_d(\text{diamond})$ can be greater than 4.5 eV. Under these circumstances <1% vacancies at the growth face is sufficient to stabilize diamond. If the effective vacancy concentration in graphite growth is slightly higher (lower) than in diamond growth, the threshold vacancy concentration would be lower (higher). For $r < 0.5$ (half as many vacancies in graphite as in diamond), vacancies would not be able to stabilize diamond even for a maximally shifted Fermi energy.

To more fully consider the growth environment, hydrogen and particularly hydrogenated vacancies might be considered. At the temperature of growth, hydrogen atoms are weakly adsorbed, however, because the desorption temperature⁷ is just above the growth temperature of 1,000 °C. Thus hydrogenated vacancies should not qualitatively affect the above analysis.

The microscopic realization of quasi-equilibrium is through kinetic processes quite similar to the realization of equilibrium. In quasi-equilibrium some processes are substantially faster than others, the slow processes not realizing equilibrium and the fast processes leading to equilibrium within accessible states. Non-equilibrium dislocations in otherwise equilibrium crystals are a well-known example. There are many kinetic processes that may lead to the imposed presence of vacancies at the growth face including aspects of existing kinetic pictures of diamond growth⁵⁻⁸. The simplest, is a picture where atoms are added and subtracted (etched) from the surface rapidly whereas vacancy hopping and surface reorganization is comparatively slow. Layers are added and subtracted as either diamond or graphite: this is the fast process in which diamond and graphite growth competition occurs. In such a picture of almost ballistic growth, the vacancy concentrations should be similar for the two competing materials.

The rate of layer addition can be estimated from the arrival rate of carbon at the surface to be 10^5 layer s^{-1} , assuming unit sticking probability for a methane partial pressure of 1 torr. This is much faster than the eventual net growth of the diamond film which is $\sim 1 \mu m h^{-1}$ or ~ 2 layer s^{-1} because the rate of etching is almost equal to the rate of addition. The layer addition and subtraction rates can be compared to the hopping rate of vacancies. In diamond at 1,200 K, using a calculated migration barrier¹² of 1.9 eV, an attempt frequency of $10^{12} s^{-1}$ and estimating the entropic prefactor as $10^{-3\pm 2}$, vacancy hopping occurs at a rate of $\sim 10^{1\pm 2} s^{-1}$. For graphite the calculated barrier¹³ is 1.6 eV and, with the same assumptions, the hopping rate is $\sim 10^{2\pm 2} s^{-1}$. Thus the layer addition and subtraction may indeed be significantly faster than vacancy annealing and the induced atomic rearrangement. After growth, vacancy hopping is fast enough so that some vacancies anneal and less-mobile vacancy aggregates should form in the bulk.

The advantage of an equilibrium picture is that predictions can be made using the few parameters controlling the equilibrium rather than the many controlling the kinetic processes. In this model the parameters n and μ are the thermodynamic variables. This quasi-equilibrium picture also relates the growth to the properties of the grown material rather than to the surface or gas-phase composition.

Some of the predictions of this theory have been verified experimentally. Sawabe and Inuzuka¹⁴ and Moustakas¹⁵ have reported a significant increase in the nucleation density of the diamond films and the growth of continuous films on polished substrates by inducing electron bombardment during growth of the films. This result could be accounted for in our theory as arising from the shifting of the Fermi level towards the conduction band which reduces the energy of formation of diamond vacancies and thus, according to equation (2), fewer vacancies are required to stabilize the diamond phase. Positive-ion conditions should also enhance growth but have not yet been reported.

A correlation between diamond growth and defect density has been established in a Raman-spectroscopy study of diamond films grown by the filament-assisted CVD of mixtures of methane and hydrogen. As the total pressure of the $CH_4 + H_2$ decreased from 100 to 15 torr the diamond component in the films increased; however, the Raman line at $1,330 cm^{-1}$, which corresponds to carbon bonded in the diamond form, broadens by approximately a factor of four without a frequency shift. Further reduction in the total pressure leads to films made of disordered carbon. A line broadening of this magnitude indicates that the diamond films contain a very high density of defects so that the phonon lifetimes are significantly reduced. If the density of these defects exceeds a critical limit the carbon films acquire a disordered structure.

The model predicts that diamond can nucleate under low pressures only under kinetic conditions that favour the formation of vacancies near the growth face. Although many of these vacancies are likely to be filled in as growth continues, a significant concentration should be present in the resulting material and these vacancies will affect the optical and electronic properties of the films. Vacancy stabilization implies that highly defect-free diamonds are difficult to grow.

Based on the conclusions of our model we propose that one way to grow diamond films with a smaller concentration of vacancies is to dope the material during the growth so that it becomes a p- or n- type semiconductor. Furthermore, the incorporation of active dopants can also be directly considered within the thermodynamic model. Because the Fermi energy can be controlled by electron bombardment, the energy for incorporation of an active dopant is also given by equation (3) where q is the charge on the active dopant $+$ ($-$) for n- (p-) type dopants. Thus the energy of the incorporated impurity is lowered and the ease of incorporation of dopants increases if the Fermi energy is forced in the opposite direction to the effect of the dopant^{10,11}. We thus propose that under electron-rich conditions

it is easier to grow p-type material whereas under electron-poor (positive ion) conditions it is easier to grow n-type material. The doping efficiency in the resulting material is then controlled by the balance of vacancies and dopants^{10,11}. These predictions need to be verified experimentally. $\square$

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Reduction of stenols to stanols in particulate matter at oxic-anoxic boundaries in sea water

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STEROIDAL alcohols (sterols) are fundamental biochemicals in eukaryotes and are indicators of organic matter sources and transformations in sea water and sediments¹⁻³. Reduction of Δ^5 -stenols, the predominant biogenic sterols, to $5\alpha(H)$ -stanols is primarily an anaerobic microbial transformation^{4,5}. Increased stanol/stenol ratios are used as evidence of this process, for example in Recent sediments where stanol/stenol ratios are higher at lower redox potential^{2,6-9}. The oxic nature of sea water generally seems to preclude this conversion¹⁰⁻¹². However, increased stanol/stenol ratios can also reflect direct biogenic input of stanols or preferential degradation of stenols relative to stanols. Here I report increased $5\alpha(H)$ -stanol/ Δ^5 -stenol ratios in particulate matter at the oxic-anoxic interfaces in the water columns of the Cariaco Trench and Black Sea and attribute them to *in situ* microbial conversion of stenol to stanol. The extent of conversion varies with water-column redox potential: little stanol generation occurs under oxic conditions, whereas there is substantial conversion in anoxic waters. These results imply that anoxic waters, particularly near oxic-anoxic interfaces, are important sites of intense alteration of organic matter.

Particle samples from four oceanic regions of differing water-column redox conditions (Fig. 1) were sampled by large-volume *in situ* filtration and analysed for sterol composition. The VERTEX IV site north of Hawaii was characterized by a weak O_2 minimum at mid-depth, whereas the VERTEX II/III site off western Mexico had a strong O_2 minimum at 100 m. The Cariaco Trench and Black Sea are both anoxic basins. The Cariaco Trench, located on the Venezuelan continental shelf, had an O_2/H_2S interface at about 275 m. The redox boundary in the Black Sea occurred at about 110 m during this cruise.

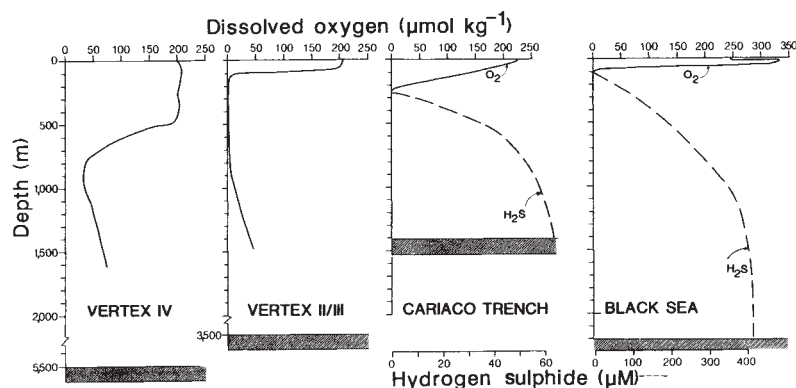


FIG. 1 Dissolved oxygen and hydrogen sulphide at the four study sites. VERTEX IV (155° W, 28° N) was sampled during July and August 1983²⁶; VERTEX II and III (16° N, 107° W) during November 1981, and October and November 1982^{27,28}; the Cariaco Trench (10°40' N, 65°30' W) during January and February 1986; Black Sea (43°10' N, 32°00' E) during cruise 5 of the Black Sea expedition in July 1988 (ref. 29).

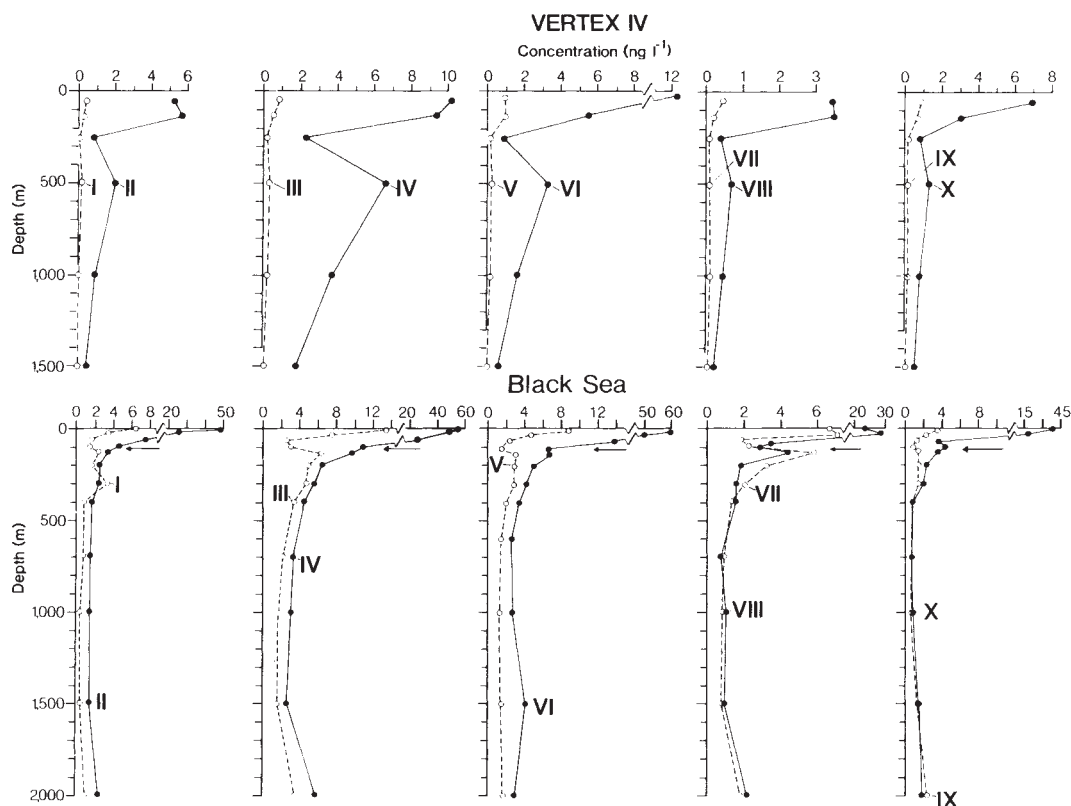
Twenty or more 4-desmethyl- and 4-methylsterols occur in the particles^{11–14}. I limit this discussion, however, to five major 4-desmethyl-5 α (H)-stanol- Δ^5 -sterol pairs (Fig. 2). 5 β (H)-stanols, also products of anaerobic reduction of Δ^5 -sterols (ref. 4), were not abundant enough to be included, and except for the Black Sea, neither were 4-methylsterols. Because particles in the Black Sea contain large amounts of some dinoflagellate-derived 4-methylsterols, especially 4 α ,23,24-trimethylcholest-22E-en-3 β -ol, it was not possible to examine the effect of the 4-methyl group on reduction of the Δ^5 -bond (such as the conversion of 4 α ,23,24-trimethylcholesta-5,22E-dien-3 β -ol to 4 α ,23,24-trimethylcholest-22E-en-3 β -ol).

Relatively high stanol concentrations (Fig. 2), low stanol concentrations and low stanol/stenol ratios (0.05–0.2; Fig. 3) in surface waters are consistent with biosynthesis of stenols, and to a lesser extent stanols, by surface-dwelling organisms^{15,16}. As depth in the oxic water column (VERTEX IV) increased, both stanol and stanol concentrations decreased, roughly in parallel,

because particle concentrations decrease and particulate organic matter decomposes as it sinks. Stanol/stenol ratios at VERTEX IV remain low down the water column. The dominant stanol source apparently is plankton living in surface waters. The lack of change in the stanol/stenol ratios indicates that there is neither conversion of Δ^5 -sterols to 5 α (H)-stanols under oxic conditions nor preferential degradation of stenols.

By contrast, stanol concentrations do not parallel profiles of the corresponding Δ^5 -sterols in suboxic (VERTEX II/III) and anoxic (Cariaco and Black Sea) waters (Fig. 2). Stanol concentrations generally increase with depth relative to stenols, in some cases being of equal to or greater than stenols in concentration. The resulting stanol/stenol ratios increase slightly in the suboxic zone at VERTEX II/III and substantially at the top of the anoxic zones (Figs 3 and 4a), in the order VERTEX II/III < Cariaco Trench < Black Sea. Thus, as in sediments, decreasing redox potentials in the water column (as reflected by O₂ and H₂S concentrations) lead to increased stanol/stenol ratios.

FIG. 2 Concentrations (ng l⁻¹) for five stanol and stanol pairs as a function of depth in the water column. Compound designations (see structures Fig. 5): 5 α (H)-cholest-22E-en-3 β -ol (I); cholesta-5,22E-dien-3 β -ol (II); 5 α (H)-cholestan-3 β -ol (III); cholest-5-en-3 β -ol (IV); 24-methyl-5 α (H)-cholest-22E-en-3 β -ol (V); 24-methylcholesta-5,22E-dien-3 β -ol (VI); 24-methyl-5 α (H)-cholest-24(28)-en-3 β -ol (VII); 24-methylcholesta-5,24(28)-dien-3 β -ol (VIII); 24-ethyl-5 α (H)-cholestan-3 β -ol (IX); 24-ethylcholest-5-en-3 β -ol (X). Arrows indicate the redox boundary for the Cariaco Trench and Black Sea. I collected particles by large-volume *in situ* filtration of 500–5,000 l of seawater¹¹. Filters were extracted with chloroform:methanol (2:1) and the lipids fractionated on silica gel. Sterol/trimethyl silyl ethers were analysed by capillary gas chromatography and gas chromatography/mass spectrometry. Depth profiles for the VERTEX II/III¹³ and Cariaco Trench samples¹⁴ are intermediate between the two shown.



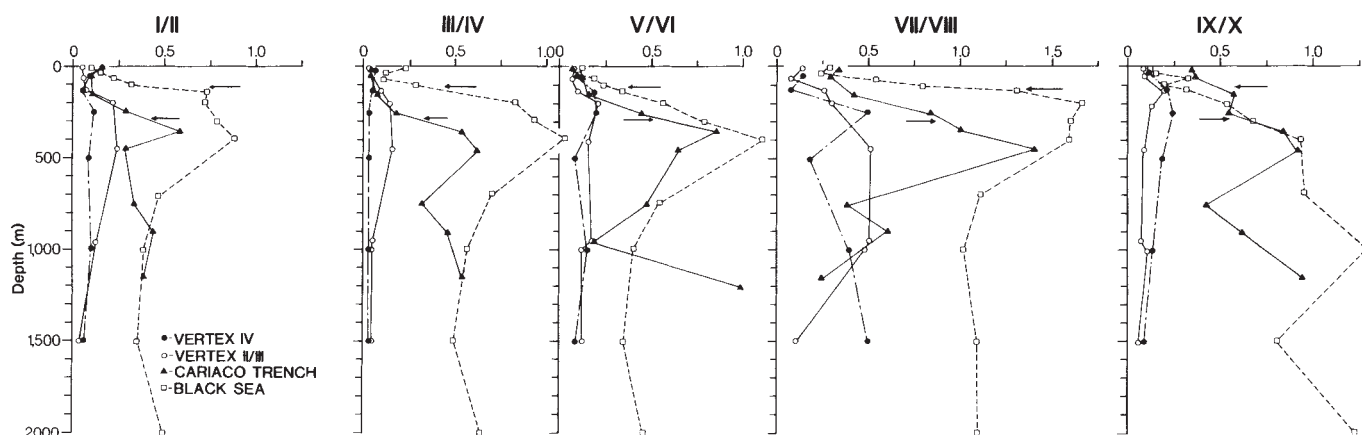


FIG. 3 Stanol/stenol ratios for the five stanol-stenol pairs shown in Fig. 2. Arrows indicate the redox boundaries.

The highest stanol/stenol ratios are consistently in the zones of maximum microbial activity (that is, the suboxic zone and immediately below the O_2/H_2S boundary¹⁷⁻²¹). This indicates a microbial role in determining the stanol/stenol distributions. Few bacteria biosynthesize sterols^{22,23}, so the bacteria are probably involved in modifying the sterol composition of particles initially derived from plankton. The ability of at least some anaerobic bacteria to convert Δ^5 -stenols to $5\alpha(H)$ -stanols (and $5\beta(H)$ -stanols as well) is well documented^{4,5}. Steroidal ketones are intermediates in stanol-stenol conversion pathways^{1,2}, but their concentrations remain low throughout the water columns studied, either because of low production rates or because they are rapidly turned over.

A chemical conversion of stenols to stanols in the systems studied is unlikely. In general, chemical transformations of marine organic matter are slower than biological reactions²⁴ and are thought to be of minor importance in stanol production in Recent sediments as well². A chemical reaction might also reduce side-chain (that is, Δ^{22}) double bonds, but there is no evidence of this occurring here or in other studies^{2,25}. Preferential degradation of stenols, either biological or chemical, would also lead to increased stanol/stenol ratios. The increased ratios observed here, however, actually result from increasing concentrations of some stanols relative to stenols rather than decreasing concentrations of stenols alone. It is also unlikely that increased concentrations of stanols, and hence increased stanol/stenol ratios, at depth result from pulsed inputs of stanol-rich particles from surface waters. Except for 4-methylstanols produced by, for example, dinoflagellates, stanols are generally not as abundant in plankton as in the particle samples at the redox boundary. It is unlikely that pulses occurred at the oxic-anoxic interfaces of both the Cariaco Trench and the Black Sea. Elevated stanol/stenol ratios in the water column probably do not result from lateral inputs of stanols produced in reducing sediments on continental margins. Surface sediments from these study sites had, for example, $5\alpha(H)$ -cholestan- 3β -ol/cholest-5-en- 3β -ol ratios of 0.15, 0.3 and 0.5 for VERTEX II/III, Cariaco Trench and Black Sea, respectively, typical values for surface sediments but much lower than in the water column.

If high stanol/stenol ratios in the Cariaco Trench and Black Sea particles are due to an *in situ* microbial stanol $\rightarrow$ stanol reduction at the oxic-anoxic interface, is the conversion rate (or more properly the extent of conversion) different for particles of different sizes? To examine the relationship between particle size and organic composition, particles from the Cariaco and Black Sea were size-fractionated *in situ* into $<53\text{-}\mu\text{m}$ and $>53\text{-}\mu\text{m}$ particles. Concentrations of sterols in the $<53\text{-}\mu\text{m}$ fraction were generally twice that of the $>53\text{-}\mu\text{m}$ fraction, for example in the Black Sea ranging from 270 ng l^{-1} and 90 ng l^{-1} for $<53\text{-}\mu\text{m}$

μm and $>53\text{-}\mu\text{m}$ particles at 30 m , to 32 ng l^{-1} and 17 ng l^{-1} at 300 m and 35 ng l^{-1} and 16 ng l^{-1} at $2,000\text{ m}$ for $<53\text{-}\mu\text{m}$ and $>53\text{-}\mu\text{m}$ particles, respectively. Consistently, the smaller particles ($<53\text{ }\mu\text{m}$) were enriched in stanols over the larger particles ($>53\text{ }\mu\text{m}$) (Fig. 4b). Similar trends were seen for all of the five stanol-stenol pairs (data not shown), so the ratios in Fig. 4b are not greatly skewed by unusual ratios of any single pair. The summed stanol/stenol ratio of the $>53\text{-}\mu\text{m}$ particles is not significantly biased by the expected abundance of zooplankton-derived cholest-5-en- 3β -ol in this size fraction relative to the $<53\text{-}\mu\text{m}$ fraction. For example, at 30 m where a direct zooplankton input could be important, $5\alpha(H)$ -cholestan- 3β -ol/cholest-5-en- 3β -ol ratios were 0.09 and 0.10 for $<53\text{-}\mu\text{m}$ and $>53\text{-}\mu\text{m}$ particles, respectively. At 400 m where a zooplankton input to the $>53\text{-}\mu\text{m}$ particles could be more important than to the $<53\text{-}\mu\text{m}$ particles, $5\alpha(H)$ -cholestan- 3β -ol/cholest-5-en- 3β -ol ratios were 1.5 and 0.22 for $<53\text{-}\mu\text{m}$ and $>53\text{-}\mu\text{m}$ particles. The same conclusion was drawn from the Cariaco Trench data set, but the comparison cannot be extended to the VERTEX IV and II/III sites as particles there were not size fractionated.

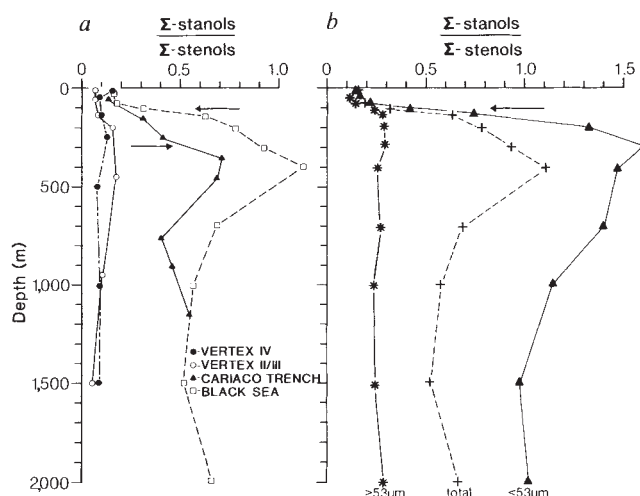


FIG. 4 a, Ratio of total stanols/total stenols (II + IV + VI + VIII + X/I + III + V + VII + IX) at the study sites. b, Total stanol/total stenol ratios for size-fractionated particles in the Black Sea ($<53\text{-}\mu\text{m}$ particles, $>53\text{-}\mu\text{m}$ particles, total ($<53 + >53\text{ }\mu\text{m}$) particles). The ratio for the total particles is the same as that shown in a. The particles were fractionated using a sandwich of a $53\text{-}\mu\text{m}$ Nitex screen over a glass-fibre filter (Type GF/F). Arrows indicate the redox boundaries.

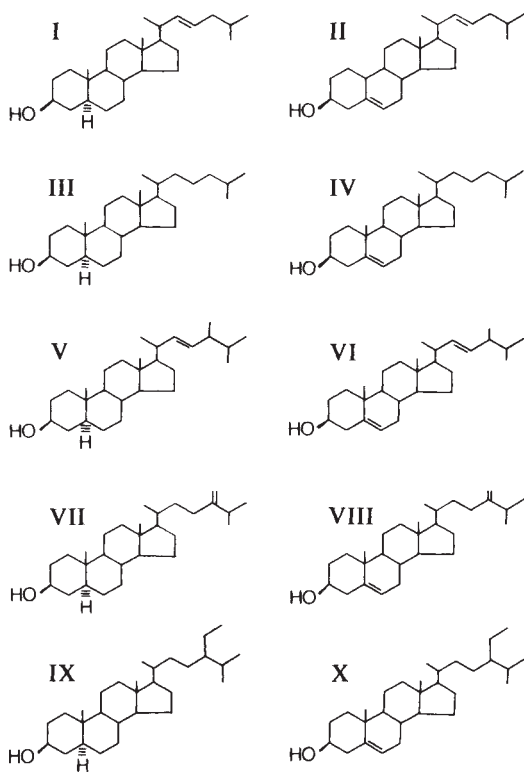


FIG. 5 Stanol and stanol structures. Compounds designated as in Fig. 2 legend.

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Liquid sulphur lakes at Poás volcano

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THE level of the hot, acidic crater lake at Volcán Poás, Costa Rica, dropped progressively over a two-year period up to April 1989, when only scattered boiling mud pools remained^{1–3}. Here we report the observation of large bodies of molten sulphur occupying part of the former lake bottom, which formed after the last of the lake water had disappeared. To our knowledge, this is the first reported occurrence of terrestrial sulphur lakes. We suggest that their formation resulted from removal of the overlying water, which allowed lake sediment temperatures to rise above the liquidus of the elemental sulphur deposits contained within them. The sulphur ponds bubbled vigorously because of the flux of hot gases from below, which kept them molten at a temperature of ~116 °C. The low viscosity of sulphur at this temperature is likely to have been critical in enabling its migration through the lake sediments to collect at fumarole vents. Some aspects of the origin of the sulphur lakes at Poás may be analogous to the putative formation of sulphur bodies on the jovian moon Io^{4,5}.

Poás is a composite volcano of the Cordillera Central in Costa Rica, rising 1,300 m above its base at 1,400 m. Historically it has been in a state of nearly continuous mild activity. Since an episode of explosive/effusive eruptions in 1953–5 it has been characterized by intense fumarole emissions and intermittent phreatic eruptions, usually through a shallow crater lake. The lake has an important role in the hydrothermal system of the volcano, typically condensing ~100 kg s⁻¹ of steam from lake-

A stanol → stanol conversion seems to occur in anoxic waters, and it seems to occur primarily in the smaller particles. The lens of anaerobic bacteria occurring at the O₂/H₂S interface of the Cariaco Trench and Black Sea^{17–21} is probably responsible. These small cells would be preferentially collected in the <53-μm fraction, producing a maximum in the stanol/stanol ratio just below the O₂/H₂S interface. The larger (>53 μm) particles, that is those having greater sinking velocities and contributing more to the vertical flux to the sediments, have lower ratios that are similar to relatively unaltered surface material. Indeed, a sediment-trap sample from 400 m in the Black Sea had a 5α(H)-cholestan-3β-ol/cholest-5-en-3β-ol ratio of 0.21, and ratios of other stanol/stanol pairs were comparably low. At the same time, however, it may be that the slowly sinking 'suspended' particles simply have a longer residence time in the region of the microbially active redox interface. Thus increased apparent conversion of Δ⁵-stanols into 5α(H)-stanols may reflect this longer residence time in the 'microbial reactor' rather than strictly higher conversion rates in the small particles.

The conclusion that Δ⁵-stanols are reduced to 5α(H)-stanols in anoxic waters contrasts sharply with an earlier report of no such conversion in the Black Sea²⁵. This discrepancy is probably due to the different sampling strategies used. The *in situ* filtering system used in this study collected particles from 1,000–5,000 l of sea water, whereas 20 l collected in standard water bottles were processed previously. It is thus likely that the two techniques sampled different portions of the particle field, thereby yielding different results.

The stanol to stanol conversion as a classical indicator of diagenesis in Recent sediments seems to work in the water column as well. Studies at redox boundaries, or other biogeochemical discontinuities, in aquatic water columns offer a unique opportunity to evaluate the role of microorganisms in organic-matter transformations. □

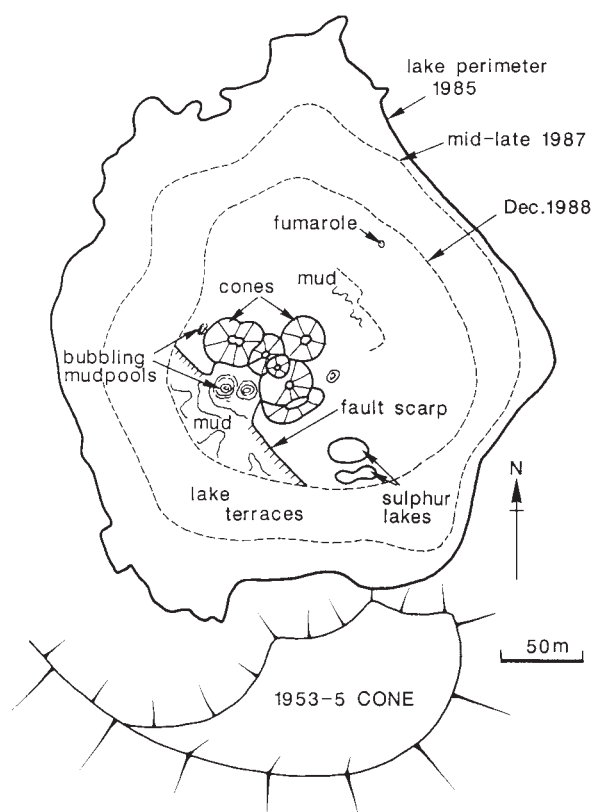


FIG. 1 Sketch map of the Poás intracrater on 23 April 1989, with former lake levels shown for comparison.

floor fumaroles and transferring heat by evaporation, radiation and conduction at the water surface (ref. 6; D.S., G. C. Brown and H. Rymer, manuscript in preparation). A shallow injection of magma beneath the lake between 1987–1988, inferred from microgravity increases³, may be responsible for its recent desiccation (Figs 1, 2).

Between early 1987 and March 1989 the water level dropped by ~32 m (ref. 1), falling the last 6 m in less than 2 months, revealing a muddy floor scattered with boiling mud pools. Two of these pools located towards the south-east part of the crater subsequently dried up and became the site of several 1–3 m high yellow cones composed of fragmental sulphur and lithic particles (Fig. 3a). Occasionally, 1-m-high chimneys developed at their apices. The cones collapsed repeatedly, forming pits that continued to emit gases. When we observed the volcano on 16 March 1989, one of these pits, ~2 m in diameter, contained a bubbling pool of brown liquid (Fig. 3b). A sample of this material quenched rapidly to vesiculated pale-green sulphur with minor admixed lake sediment. Qualitative X-ray fluorescence analysis has since confirmed the purity of the sulphur, although significant traces of selenium and arsenic were detected.

By 19 April 1989, following a period of continual modification of the cones (Fig. 3c–e), two larger sulphur lakes were established side by side within steep-sided collapse pits. One had a dumbbell-shaped perimeter with maximum dimensions ~24 × 11 m; the other was roughly elliptical with axes ~28 × 15 m (Figs 2, 3e). When the crater was next visited on 23 April 1989, the sulphur-pond surface had dropped about 0.5 m and was >1 m below the rim of each pit. Blocks of sediment from the pit walls seemed to float on the liquid, which was dark brown in colour, visibly translucent and remarkably fluid, bubbling vigorously over most of its surface. The temperature of the molten sulphur, measured by thermocouple, was 116 °C (J. Barquero, personal

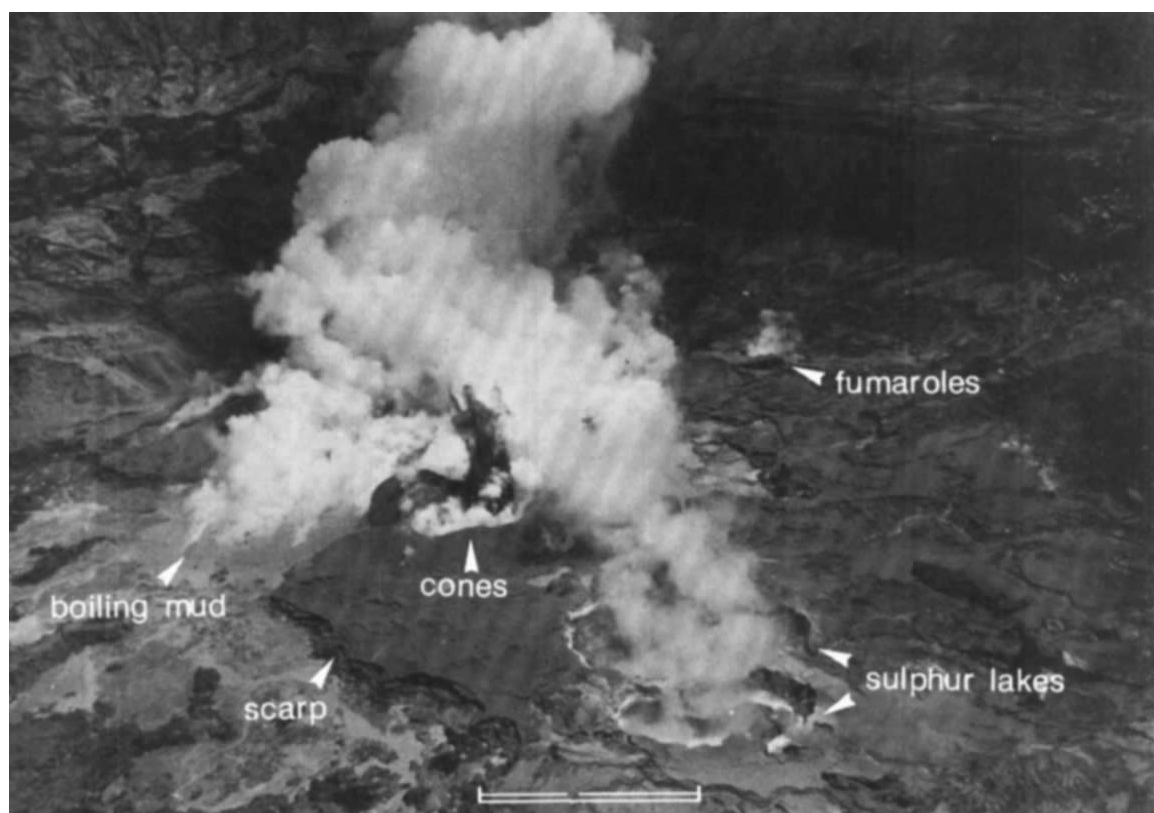


FIG. 2 Photograph of desiccated lake floor (on 23 April 1989) taken from eastern end of the 1953–5 cone, looking approximately north-west. Scale

bar is ~25 m.

communication), well below the boiling point of sulphur ($\sim 444^\circ\text{C}$). There was no crust on the surface although a terrace of pale-yellow solid sulphur had formed around the edge, and pit walls and rims were coated in bright-yellow sulphur sublimates. Clouds of condensed steam rose above the liquid surface and the smell of sulphur dioxide was strong. These observations are important as they indicate that the sulphur was kept molten and uncrusted by the passage of hot gases (presumably at a temperature close to 116°C). Meanwhile, 50–100 m away, spectacular phreatic emissions of non-juvenile ash and blocks were building a cluster of at least six cones, about 10 m high (ref. 2) (Figs 1 and 2). Some of the plumes were dark and cypressoid; others were yellow, presumably because they contained elemental sulphur.

To our knowledge, terrestrial sulphur lakes have not been documented before. 'Steaming pits' and 'niches' containing liquid sulphur were reported⁷ at Ebeko volcano (Kuriles, USSR) in 1952 but, unfortunately, their dimensions were not recorded. These features may have been nothing more unusual than the dribbles of molten sulphur commonly found at fumarole fields and solfataras. However, Ebeko did produce a sulphur flow in 1934⁸ and similar flows have also been recorded at Siretoko-Iôsan (Japan)⁹, Volcan Azufre (Galapagos Islands)¹⁰, Mauna Loa (Hawaii)^{11,12} and Lastarria (Chile)¹³. These rare occurrences of flows of molten sulphur have been attributed^{13,14} to melting and mobilization of sulphur deposits of fumarolic origin.

Although sulphur lakes were hitherto unknown, their existence beneath aqueous crater lakes has been inferred from observations of floating sulphur 'spherules' found at the volcanoes Kusatsu-Shirane (Japan)¹⁵ and Ruapehu (New Zealand)¹⁶. Ohashi proposed¹⁵ that the hollow spherules were formed by the upwards passage of gas bubbles through liquid sulphur, and manufactured similar morphologies in a laboratory experiment. Sulphur 'bubbles' have commonly been observed¹⁷ floating on the lake water at Poás but, here, subaqueous sulphur

lakes were postulated on different grounds—the recovery¹⁸ of greenish sulphur pyroclasts ejected by phreatic emissions through the crater lake during the 1970s¹⁹. The morphology of the sulphur ejecta^{18,19}, as in the case of the spherules, indicated formation in the liquid phase. Francis *et al.* suggested¹⁹ that melting of sulphur sublimates in large volumes of rock surrounding fumaroles had taken place at Poás. The liquid then accumulated in fissures and pore spaces before being sprayed into the air by explosive eruptions, such as those in September 1978 (when, perhaps significantly, the lake floor was partially exposed¹⁹).

We suppose that the liquid sulphur ponds at Poás were also supplied by remobilized fumarolic deposits (although a primary melt separated from silicate magma at depth is feasible^{20,21}) on the grounds that secondary sulphur deposits are abundant at Poás. They occur within the lake sediments^{22,23}, as disseminated sulphur particles and as sulphur-filled fumarole pipes and tubes. An eruption in January 1988 ejected lake mud with ~ 5 –10 vol% sulphur particles (G. Brown, personal communication). The oxidation of H_2S by SO_2 in the acidic aqueous conditions of the crater lake¹⁶ may be responsible for their formation. The reaction rapidly precipitates elemental sulphur which would settle on the lake bed. Both H_2S and SO_2 have been identified at Poás: gas analyses⁸ of fumaroles on the near-dry crater floor in February 1989 showed 5.0 mol% H_2S , and the SO_2 flux, measured²⁰ by correlation spectroscopy in February 1982, was $\sim 700 \text{ t d}^{-1}$.

The presence or absence of water must have an important role in the change of state of sulphur. Although there may be localized pockets of molten sulphur where gas temperatures exceed the sulphur liquidus, widespread melting will be inhibited when the aqueous lake is present because the temperature of the water-saturated sediments remains below the local boiling point of water. The loss of the water removes this temperature buffering system so that temperatures of sediment

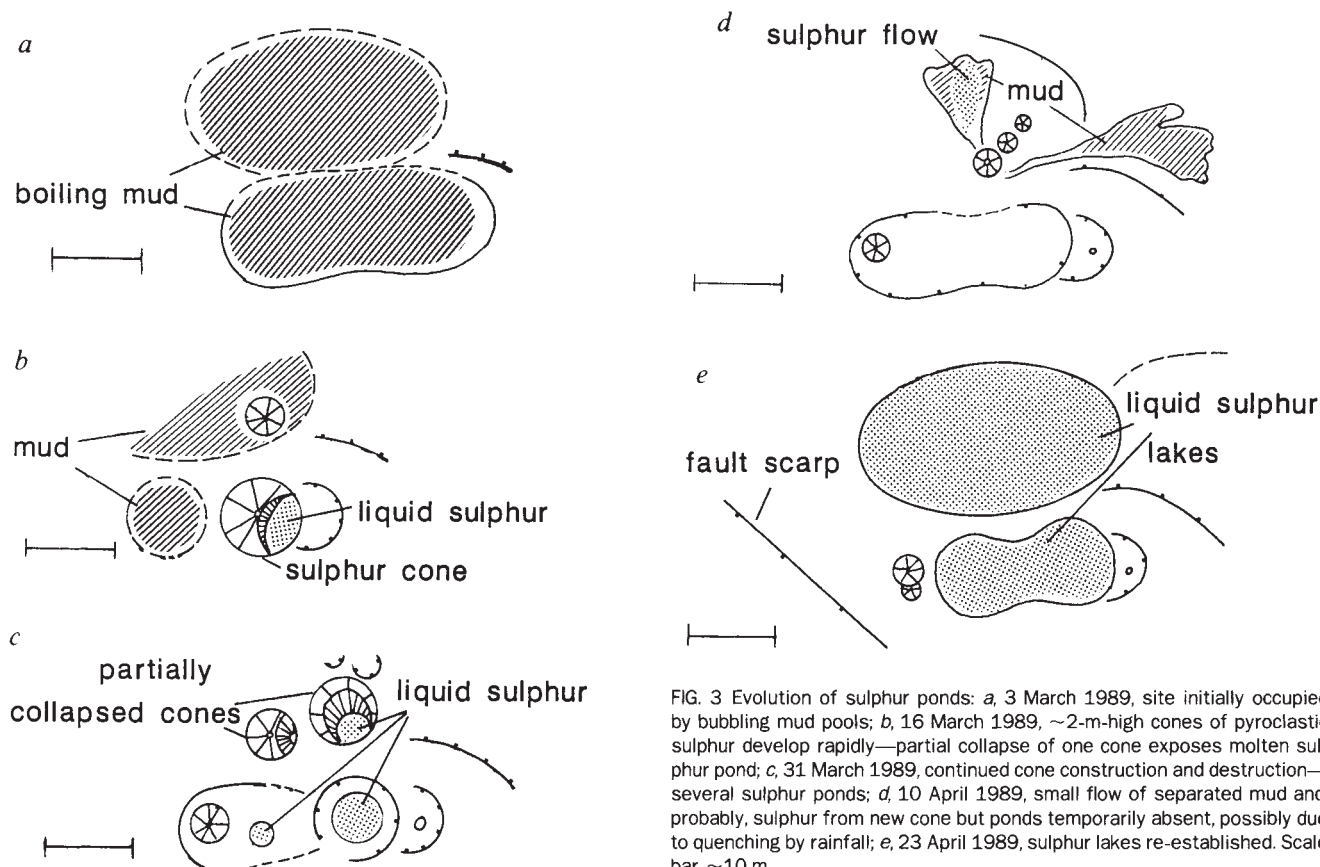


FIG. 3 Evolution of sulphur ponds: a, 3 March 1989, site initially occupied by bubbling mud pools; b, 16 March 1989, ~ 2 -m-high cones of pyroclastic sulphur develop rapidly—partial collapse of one cone exposes molten sulphur pond; c, 31 March 1989, continued cone construction and destruction—several sulphur ponds; d, 10 April 1989, small flow of separated mud and, probably, sulphur from new cone but ponds temporarily absent, possibly due to quenching by rainfall; e, 23 April 1989, sulphur lakes re-established. Scale bar ~ 10 m.

fluxed by hot gases can increase above the sulphur liquidus, generating much greater volumes of melt. The Poás sulphur ponds did not appear until all the water in the vicinity had been driven off, and then following a period of sulphur-cone construction and destruction. The collapse of the cones into molten sulphur pools indicates that chambers of the liquid may have developed at very shallow levels beneath the lake bed.

Concentration of the molten sulphur at these specific sites is more difficult to explain. There is no obvious topographic cause, as seemed to be the case of the Mauna Loa sulphur flow¹². However, in the absence of data on the volume of the sulphur lakes at Poás, or of the proportion of sulphur contained in the sediments, it is not possible to estimate the region over which the sulphur has migrated. Clearly, proximity to fumarolic vents is important as indicated by the gas flux through the sulphur lakes but the pond temperature of 116 °C is also highly significant. Between its melting point, ~113 °C, and ~159 °C, at which cyclic octatomic sulphur begins to polymerize, the viscosity of liquid sulphur is of the order of only 10 cp (refs 13, 14), about ten times that of liquid water at room temperature⁴. Such low viscosity is likely to have been a critical factor enabling the molten sulphur to move towards fumarole sites through fractures in the lake sediments or through pores created by melting of the originally disseminated sulphur.

Terrestrial sulphur flows received renewed attention following recognition of the importance of sulphur volcanism on Io, from the images of the Voyager spacecraft. For example, Greeley *et al.*¹² re-examined the Mauna Loa sulphur flow to gain insight on possible processes taking place on Io. Unfortunately, the evidence for liquid sulphur bodies on Io is inconclusive: (1) ground-based and Voyager observations in the infrared identified ~130–430 °C hotspots, temperatures that were argued^{24,25} to be too low for silicate magma. It is well known, however, that lava bodies are typically composed of cool crust as well as glowing cracks and indeed temperatures of terrestrial lava surfaces derived from single-channel satellite data²⁶ fall in the same range. (We measured a peak radiant temperature of 97.0 °C for the surface of the Poás sulphur ponds with an 8–14 µm bandpass radiometer, the low value is probably due to the attenuation of emitted infrared by fumes); (2) the reflectance spectra of volcanic regions of Io have been related to those of elemental sulphur, the colour variations of which with temperature changes are well known^{12,27}. More recently, however, Hapke has argued²⁸ that the only unambiguously identified species is SO₂, and that all the observed spectra can be modelled by a combination of SO₂ condensates and basalt.

Because of this controversy, we make only tentative implications regarding Io from our observations at Poás. The most interesting and instructive parallel concerns the role of water. Unlike the other galilean satellites, Io has lost its water, and so S_n and SO₂ are likely to be the dominant volcanic volatiles⁴—sulphur volcanism on Io takes place in the absence of water. The appearance of sulphur lakes at Poás following desiccation of the aqueous crater lake may therefore mimic to some extent the evolution of sulphur volcanism on Io. The low viscosity, as observed at Poás, is likely to be a significant factor in coalescing large volumes of molten sulphur within a solid matrix. □

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Role of methane and carbon dioxide in gold deposition

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VEIN-HOSTED gold deposits in low- to medium-grade metamorphic terrains are commonly associated with low-salinity hydrothermal fluids rich in CO₂ and/or CH₄^{1–12}. Fluid inclusion studies of gold mineralization indicate that the ore fluid comprised co-existing CO₂/CH₄-rich and H₂O-rich phases, and that phase separation played an integral part in gold deposition^{3,5,6,8–12}. In hydrothermal solution gold is present as the Au(HS)₂[–] complex¹³. Precipitation of gold caused by decreasing ligand activity involving the formation of iron sulphides from wall-rock iron oxides and silicates¹³ is clearly relevant to gold deposits associated with iron formations and iron-rich igneous rock^{14,15}. It cannot, however, be used to explain the common association of gold deposits with black shales or schists^{3,5,7,8,16}, where wall-rock iron is in the form of sulphides, and therefore generally in equilibrium with the hydrothermal fluid, or with granitoids or felsic volcanics, where the amount of iron is low. This latter association may be explained by the partitioning of H₂S into the non-aqueous phase during fluid immiscibility^{10,12}, but the general applicability of this mechanism is not known. Here we present a new synthesis of experimental data from a variety of sources which puts this mechanism on a semi-quantitative basis, and suggest that it may be applicable to a wide variety of hydrothermal gold environments.

Recent high-pressure (*P*)-high-temperature (*T*) experimental studies of the systems H₂O–CO₂–NaCl (ref. 17), H₂O–CH₄–NaCl (refs 18, 19) and H₂O–H₂S–CH₄–CO₂ (refs 20, 21) enable modelling of fluid unmixing in carbonic/methanoic ore fluids. First, in the system H₂O–H₂S–CH₄–CO₂ at the two-phase boundary, hydrogen sulphide is strongly partitioned into the vapour phase (Fig. 1). For an initial homogeneous ore fluid, the separation of hydrogen sulphide into the non-aqueous, carbon dioxide/methane-rich vapour phase would result in mass transfer of sulphur from the aqueous component of the hydrothermal fluid to the non-aqueous phase with concomitant deposition of gold. Moreover, because the ratio H₂S(v)/H₂S(l) (in terms of orders of magnitude) is largely independent of pressure, temperature and composition, at least up to 200 °C and 200 bar, this mechanism may be valid for a wide range of geological environments. At higher temperatures and pressures, particularly for compositions close to the critical curve, the partition coefficient for hydrogen sulphide between the aqueous and non-aqueous phases will approach unity. In view of the flattened form of the solvus, however, the upper part of the two-phase region corresponds to a restricted *P*–*T* geological domain.

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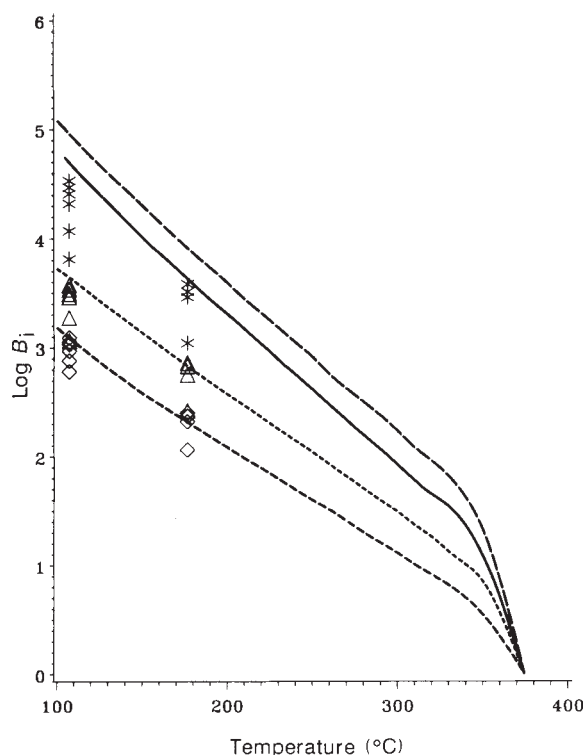


FIG. 1 Diagram illustrating the variation in $\log B_i$ (where B_i is the ratio of concentration of gas in the vapour phase to gas in the liquid phase in molal concentration units) with temperature for various gases in pure water at the vapour pressure of water (solid line, CH_4 ; short-dashed line, CO_2 ; medium-dashed line, H_2S ; long-dashed line, N_2). Also shown are the data for two carbonic fluids with compositions: 50 mol% H_2O , 15 mol% CH_4 , 30 mol% CO_2 , 5 mol% H_2S and 50 mol% H_2O , 5 mol% CH_4 , 5 mol% CO_2 , 40% H_2S at pressures up to 180 bar (stars, CH_4 ; triangles, CO_2 ; diamonds, H_2S). Over a wide temperature range H_2S is strongly (>90%) partitioned into the vapour phase irrespective of the initial composition of the original fluid. Data for the water system is from ref. 21 and data for the CH_4 - CO_2 - H_2S - H_2O system is from ref. 20.

Second, the experimental data describe the conditions under which optimum fluid unmixing will take place. In the system H_2O - CH_4 - NaCl (Fig. 2), only small amounts of methane (≤ 5 mol%) are soluble in aqueous brines over a wide range of pressures and temperatures (up to 3 kbar and 400 °C), whereas in the system H_2O - CO_2 - NaCl (Fig. 3), significantly more carbon dioxide (up to 15 mol%) is soluble under the same conditions. Fluid inclusion studies of black-shale-hosted gold deposits^{3,5,7,8,16} frequently refer to the development of CO_2 - CH_4 -rich and aqueous-rich inclusions, and although it cannot be proved in all instances that the association represents the trapping of an immiscible fluid^{7,22}, the presence of methanoid inclusions ($\text{CH}_4/\text{CO}_2 > 1$) and the large field of fluid immiscibility in the system H_2O - CH_4 - NaCl strongly indicate some degree of unmixing during mineralization. The methane in these inclusions is thought to have been generated by reaction of the mineralizing fluid with graphite⁵, $\text{C} + 2\text{H}_2\text{O} \rightleftharpoons \text{CH}_4 + 2[\text{O}]$, where $[\text{O}]$ represents oxygen bound in carbon dioxide and/or carbonate. (^{13}C isotope data for co-existing carbonate and graphite coupled with high CH_4/CO_2 ratios in fluid inclusions from quartz adjacent to graphite in wall rocks indicate that reactions of this type do occur²³, and carbonates are a common gangue mineral in gold deposits^{1,2,4,24,25}.) The restricted solubility of methane in aqueous brines implies that very little fluid-rock interaction is required to initiate fluid immiscibility.

An analogous reaction can be written for the generation of carbon dioxide, $\text{C} + 2\text{H}_2\text{O} \rightleftharpoons \text{CO}_2 + 4[\text{H}]$, where $[\text{H}]$ represents hydrogen bound in methane and/or phyllosilicates. Hydrogen metasomatism of the wall rocks and the development of sericite,

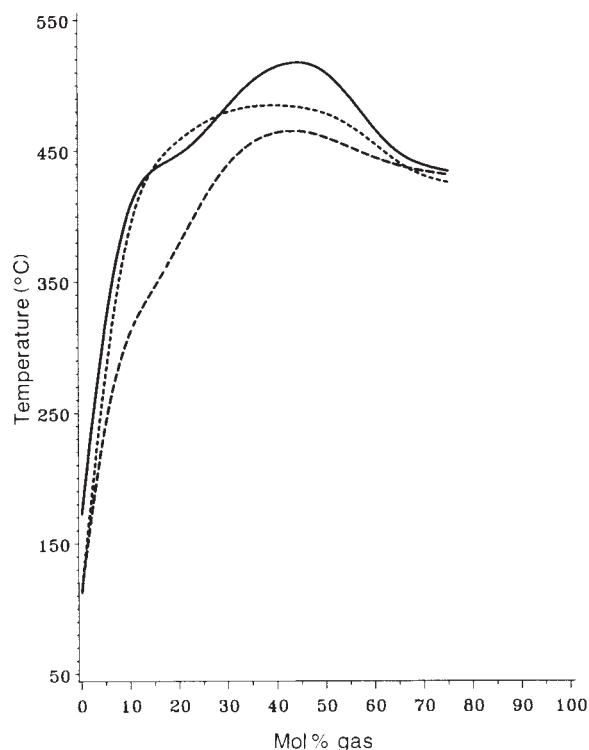
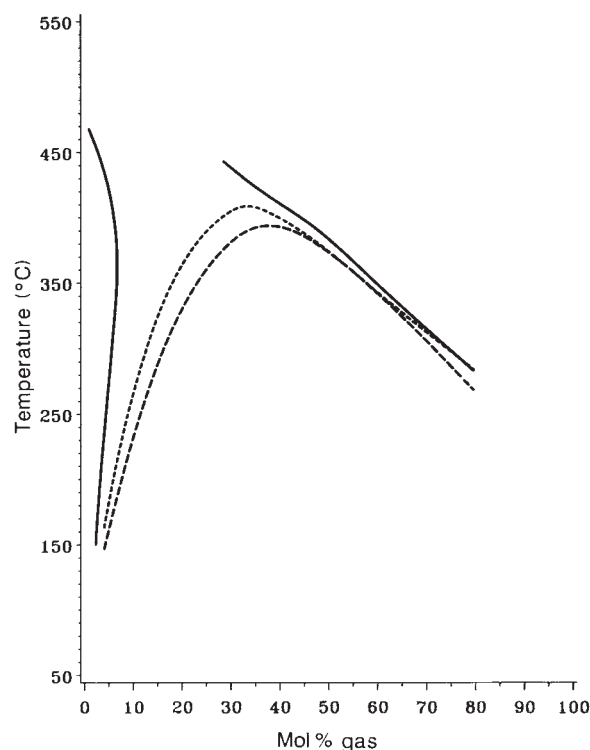


FIG. 2 Pseudobinary temperature-composition section for the system CH_4 - H_2O - NaCl (8 wt%) at 0.5 kbar (solid line), 1.5 kbar (short-dashed line) and 3.0 kbar (medium-dashed line); at all three pressures the two-phase region lies below the line. Note that at temperatures <350 °C an increase of the pressure from 0.5 kbar to 1.5 kbar (solid and short-dashed lines) only increases the dissolved gas content by a few mole per cent. Hence, changes in pressure in this P - T range only have a limited effect on methane solubility. Data from refs 18, 19.

albeit sometimes restricted, is common to many gold deposits^{5,24-26}. As the relative solubility of carbon dioxide is much higher than methane at elevated temperatures and pressures (Fig. 4) the extent of reaction needed to initiate immiscibility will be that much greater. Thus in the P - T range under consideration, fluid immiscibility and the precipitation of gold is favoured by wall-rock reactions with graphite that produce methane as the dominant volatile species (that is, under reducing conditions where the ambient f_{O_2} is low). Analysis of fluid inclusion volatiles associated with black-shale-hosted gold mineralization supports this hypothesis, as shown by the positive correlation between high CH_4/CO_2 ratios and gold grade^{5,27}.

Thus, the partitioning of hydrogen sulphide into the vapour phase during the separation of a methane-rich vapour phase constitutes a very powerful mechanism for bringing about gold deposition. The extensive two-phase field for the system H_2O - CH_4 - NaCl (Fig. 2) indicates that fluids containing more than a few mole per cent methane will experience significant unmixing over a wide range of geological conditions. We therefore expect that P - T conditions favourable for the formation of black-shale-hosted gold deposits characterized by methanoid fluids will be equally large. By contrast, where the hydrothermal fluid contains carbon dioxide as the dominant dissolved gas, unmixing will take place over a more restricted P - T range. For example (see Fig. 4), at 300 °C and 1.5 kbar, a H_2O - CO_2 - NaCl fluid will unmix when the carbon dioxide content of the fluid exceeds 15 mol%, whereas the corresponding H_2O - CH_4 - NaCl fluid only requires the dissolved gas content to be greater than 5 mol% for fluid unmixing to take place. This may explain the genesis of the Archaean 'greenstone'-type gold deposits the fluids of which seem to have been exceptionally rich in carbon dioxide^{1,4,11}. Gold-bearing hydrothermal fluids that contain only minor

FIG. 3 Pseudobinary temperature-composition section for the system CO_2 - H_2O -NaCl (6 wt%) at 0.5 kbar (solid line), 1.5 kbar (short-dashed line) and 3.0 kbar (medium-dashed line). At pressures ≤ 1 kbar, the solvus (solid line) splits into two portions separated by a two-phase (liquid and vapour) zone. Between 1.5 and 3.0 kbar the two-phase region lies below the line. Note that at temperatures $< 350^\circ\text{C}$ increasing the pressure from 1.5 to 3.0 kbar as with methane only increases the dissolved gas content by a few mole per cent. Thus, as for methane, increases in pressure only have a limited effect on carbon dioxide solubility in this P - T domain. At any given pressure and temperature carbon dioxide is always more soluble in low-salinity aqueous brines. Data from ref. 17.



amounts (< 5 mol%) of carbon dioxide as the major dissolved gas are unlikely to form major gold deposits in this P - T window ($1\text{--}3$ kbar and $250\text{--}400^\circ\text{C}$) as a result of fluid unmixing alone. When such fluids reach higher levels in the crust ($P < 1$ kbar), however, fluid separation will occur and would be accompanied by the deposition of gold (Fig. 3). This is the domain of epithermal gold deposits and present-day active geothermal systems.

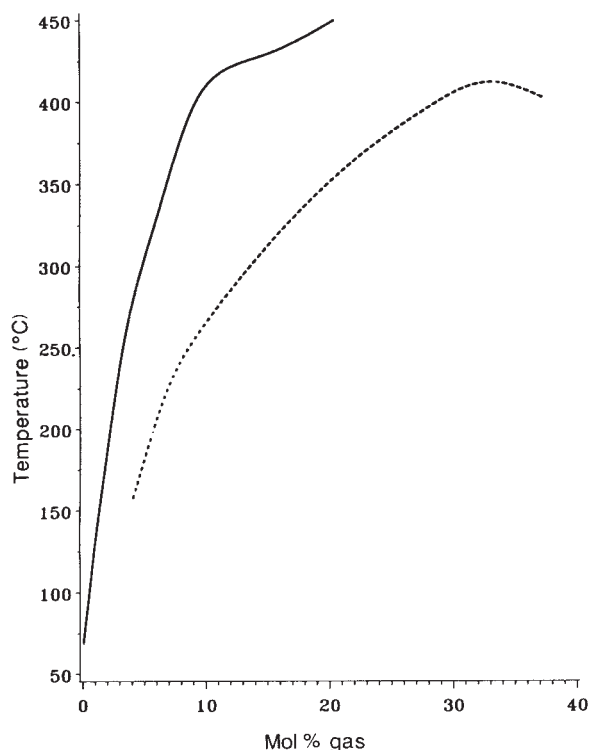


FIG. 4 Liquid-vapour curves for methane and carbon dioxide at 1.5 kbar showing the relationship between gas solubility and temperature at a pressure compatible with gold deposition in many low- to medium-grade metamorphic terrains (Solid line, CH_4 ; short-dashed line, CO_2).

These deposits commonly show evidence of 'boiling' and fluid-inclusion analysis often reveals the presence of minor amounts of carbon dioxide associated with the mineralizing fluid²⁸⁻³¹.

We believe that the hypothesis of gold deposition brought about by the partitioning of hydrogen sulphide into the vapour phase or nonaqueous ($\text{CH}_4 + \text{CO}_2$)-rich phase during fluid unmixing provides a common link between the formation of gold deposits at different structural levels in the Earth's crust. Furthermore, compared to carbon dioxide, the addition of small amounts of methane dramatically enlarges the pressure-temperature field immiscibility and will act as a powerful trigger for gold deposition. □

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Worker policing in the honeybee

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In most species of social Hymenoptera with queen-worker dimorphism, workers cannot mate but retain functional ovaries¹; because males arise from unfertilized haploid eggs, workers can potentially produce males. Worker-derived males are frequent in some species, but in others occur only in queenless colonies^{2,3}. Workers are more related to their own sons (coefficient of 0.5) than to the queen's sons (their brothers; 0.25); they are also more related to nephews (0.375) than brothers if queens mate with one male, but if queens mate with more than two unrelated males a worker's mean relatedness to nephews is less than to brothers³⁻⁵. In this case workers could benefit by 'worker policing'^{3,5}: preventing each other from producing males, perhaps by destroying worker-laid eggs or by aggression toward reproductive workers. Worker reproduction is rare in queenright colonies of species with multiply mated queens (such as honeybees⁶ and some yellowjacket wasps⁷), but is common in some monandrous species (bumblebees and stingless bees³). Here we describe experiments showing strong discrimination by honeybee (*Apis mellifera*) workers against worker-laid male eggs, supporting the worker-policing hypothesis. The honeybee was studied because queens mate with 10–20 males⁸, making worker policing seem likely as a cause for the rarity of worker-derived males (about one in a thousand males is worker-derived⁶).

We introduced queen- and worker-laid (from queenless colonies) male eggs to queenright colonies, and compared the numbers removed over 24 h (2×2 χ^2 test for independence of egg source and removal). Colonies used had naturally mated queens, were of mixed European races, and were rearing drones. In experiments 1 and 2, colonies were divided with the queenless part above the queenright part, separated by two screens 1 cm apart, to minimize colony odour differences but prevent exchange of bees, food, or queen pheromones. The two parts had entrances facing opposite directions, and were moved at the time of division to a new apiary, so that bees would orient to their own part. Once workers in the queenless part began to lay, male eggs were obtained by introducing combs of drone cells to each part. (Queens were caged on these combs using queen excluder mesh, which allows workers but not the larger queen to pass through; these queen-laid eggs were apparently all male, see Table 1). After 24 hours, combs were removed and, using special forceps^{9,10}, queen-laid eggs were removed and replaced with either another queen-laid egg from the same comb,

or, in alternate adjacent rows, with a worker-laid egg from the queenless part. The comb with transferred eggs was then put into the brood area of a queenright colony, but separated from the queen by queen-excluder mesh to prevent the queen removing or depositing eggs, and scored for egg removal 24 hours later.

Experiment 1 compared removal of mother-queen-laid and sister-worker-laid eggs (that is, eggs were reintroduced to the queenright part of their source colony). Workers accurately discriminated between these classes of eggs (Table 1): only 4 of 204 worker-laid eggs (2%), but 150 of 237 queen-laid eggs (61%) remained ($P < 0.0001$) after 24 hours.

This discrimination against worker-laid eggs could result from assessment of relatedness to eggs, preference for queen-laid over worker-laid eggs, or differences in colony odor despite the double screen. To differentiate between these, experiment 2 compared the treatment of eggs laid by the queen and by her worker daughters, all unrelated to discriminator bees (that is eggs were introduced to an unrelated colony). The results were as in experiment 1, with 59% of queen-laid and <1% of worker-laid eggs remaining (Table 1.2), indicating that the caste of the egg's mother was responsible for differential removal. In trial 4 of experiment 2, inspections were made more frequently. Removal of worker-laid eggs was rapid, with half removed in 2 hours and 90% in 6 hours (Fig. 1).

In experiment 3, an additional 11 colonies were tested using similar methods, except that queenless colonies were not kept above queenright colonies, queen- and worker-laid eggs used in a trial were not always from the same source colony, and not all eggs were transferred to a cell which previously held a queen-laid male egg. All colonies showed significantly greater removal of worker-laid eggs, except one which removed both worker- and queen-laid eggs (Table 1.3).

If worker eggs were not viable, this could explain the differential removal. However, queenless honey bee colonies often rear many males¹¹ so at least a proportion of worker-laid eggs must be viable. We compared the *in vitro* viability of queen- and worker-laid eggs by transferring 0–24-hour-old eggs of each type into a beeswax-lined Petri dish also containing a water-saturated piece of cotton, incubating them at 35 °C for four days, and comparing the numbers hatching. In five trials, 81 of 207 worker-laid eggs (39%) and 86 of 215 queen-laid eggs (40%) hatched ($P = 0.90$). (These viabilities are probably abnormally low because of dehydration or damage during transfer and incubation.). Once hatched, there seems to be no discrimination between queen- and worker-derived larvae. We transferred one-day-old larvae from their natal combs, following the methods of experiment 2 (using a 'grafting' tool as in commercial queen-rearing¹²). In one trial 47 of 57 queen derived larvae (82%), and 24 of 33 worker derived larvae (73%) remained after 24 hours ($P = 0.70$).

These results provide strong support for the worker-policing

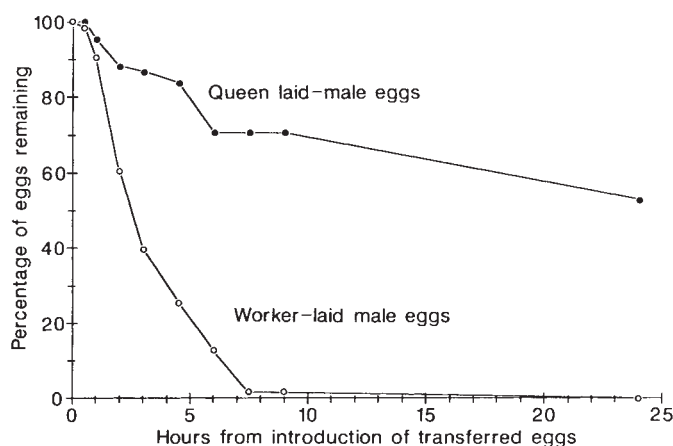


FIG. 1 Time course of egg removal for worker- and queen-laid male eggs within the brood area of a populous queenright honeybee colony. (Data from trial 4, experiment 2.)

TABLE 1 Worker policing of eggs in honey bee colonies.

Date	Source Colony W	Q	Rearing colony	Queen-laid N ₁ /N ₀	%	Worker-laid N ₁ /N ₀	%	P
Expt 1: eggs reintroduced to queenright part of colony from which eggs came								
28 March 1989	A	A	A	5/32	16%	0/33	0%	0.028
*31 March 1989	B	B	B	56/89	63%	0/60	0%	<0.0001
31 March 1989	C	C	C	23/34	68%	1/34	3%	<0.0001
1 April 1989	A	A	A	31/40	78%	3/44	7%	<0.0001
3 April 1989	B	B	B	35/42	83%	0/33	0%	<0.0001
Expt 2: eggs introduced to queenright colony unrelated to egg-source colony								
*23 March 1989	C	C	D	30/96	31%	1/88	1%	<0.0001
1 April 1989	A	A	C	39/41	95%	0/31	0%	<0.0001
6 April 1989	B	B	D	47/51	92%	0/44	0%	<0.0001
7 April 1989	B	B	C	36/68	53%	0/63	0%	<0.0001
Expt 3: worker-laid eggs and queen-laid eggs from separated colonies								
*21 June 1989	E	F	L	50/59	85%	4/45	9%	<0.0001
*21 June 1989	E	F	M	29/58	50%	0/48	0%	<0.0001
*21 June 1989	E	F	N	3/46	7%	0/52	0%	0.07 NS
23 June 1989	G	G	O	8/46	17%	0/48	0%	0.006
24 June 1989	G	G	G	4/46	9%	0/48	0%	0.045
24 June 1989	G	G	F	16/46	35%	0/46	0%	0.0002
*26 June 1989	H	F	G	25/42	60%	0/43	0%	<0.0001
*26 June 1989	G	G	P	25/58	43%	0/48	0%	<0.0001
*26 June 1989	G	G	R	7/58	12%	0/60	0%	0.0089
*5 June 1988	H	I	S	19/66	29%	0/67	0%	<0.0001
*5 June 1988	J	K	T	29/69	42%	0/71	0%	<0.0001
*5 June 1988	H	I	U	42/65	65%	0/119	0%	<0.0001
*5 June 1988	J	K	U	31/128	24%	0/72	0%	<0.0001
*1 June 1988	J	I	T	59/104	57%	0/73	0%	<0.0001
*1 June 1988	J	I	U	12/77	16%	0/70	0%	0.0014
TOTAL				661/1461	45%	9/1340	1%	

All trials resulted in significantly ($P < 0.05$) higher removal of worker-laid male eggs than queen-laid male eggs in queenright honey bee colonies, except colony N, in which both classes were removed. Letters A-U (excluding Q) designate different, unrelated honey bee colonies. For each type, N₁ is the number of eggs remaining after 24 hours, and N₀ is the number of eggs introduced. P is the alpha level of a chi-squared test for independence of treatment by the rearing colony and caste of the eggs' mothers. Trials marked * refer to cases in which brood were observed to pupation. In all such cases queen-laid eggs gave rise only to males. Experiments were conducted in Florida (March/April) and New York (June).

hypothesis. Effective removal of worker-laid male eggs occurred in all colonies. In experiments 1 to 3 only 0.7% worker-laid versus 45.2% queen-laid male eggs remained after 24 hours. Since the egg stage lasts three days, actual removal of worker-laid eggs could be still greater.

The results also allow inferences concerning the cue used in discrimination. Worker-laid eggs, but not worker-derived male larvae, are strongly discriminated against, suggesting that cues are found on the egg itself or possibly on the inner surface of the cell. The latter idea is rejected because worker-laid eggs transferred into cells previously containing queen-laid male eggs were removed (experiments 1 and 2). Because differential removal of worker-laid male eggs is not due to relatedness differences (experiments 2 and 3) the idea that there is a queen-specific egg-marketing pheromone by which workers can determine maternal origin of the egg^{3,13} is supported. Such a pheromone could evolve because both the signal¹⁴-producing queen and the signal-receiving police workers would benefit by

increased relatedness to the colony's males³. (A mechanism would also be required to detect and prevent workers cheating by producing the pheromone.) Ultimately, worker policing is based on relatedness-based discrimination, but cues or signals of maternal origin, not relatedness differences, are apparently used proximally.

Worker policing may be important in the evolution of highly cooperative worker behavior. Intra-colony conflict is inherent in insect societies because of genetic differences between colony members^{4,13,15}. However, effective worker policing causes male-production to reach a state of forced cooperation, with workers effectively sterile⁶, and the queen the sole reproductive by default³. By denying individual workers the alternative of 'selfish' reproduction, in which they could increase their inclusive fitness by manipulating colony resources, worker policing aligns the genetic interests of all workers and the queen, enhancing selection for workers to increase their inclusive fitness by increasing colony resources through cooperation. □

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Expression of an insulin-regulatable glucose carrier in muscle and fat endothelial cells

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INSULIN rapidly stimulates glucose use in the major target tissues, muscle and fat, by modulating a tissue-specific glucose transporter isoform¹⁻⁶. Access of glucose to the target tissue is restricted by endothelial cells which line the walls of nonfenestrated capillaries of fat and muscle⁷. Thus, we examined whether the capillary endothelial cells are actively involved in the modulation of glucose availability by these tissues. We report here the abundant expression of the muscle/fat glucose transporter isoform in endothelial cells, using an immunocytochemical analysis with a monoclonal antibody specific for this isoform¹. This expression is restricted to endothelial cells from the major insulin target tissues, and it is not detected in brain and liver where insulin does not activate glucose transport. The expression of the muscle/fat transporter isoform in endothelial cells is significantly greater than in the neighbouring muscle and fat cells. Following administration of insulin to animals *in vivo*, there occurs a rapid increase in the number of muscle/fat transporters present in the luminal plasma membrane of the capillary endothelial cells. These results document that insulin promotes the translocation of the muscle/fat glucose transporter in endothelial cells. It is therefore likely that endothelial cells play an important role in the regulation of glucose use by the major insulin target tissues in normal and diseased states.

To determine the possible role of endothelial cells from insulin-sensitive tissues in the regulation of glucose use, we performed immunocytochemical studies using two monoclonal antibodies of different specificity: antibody 1F8 which recognizes the muscle/fat (M) transporter isoform¹ and antibody B315:32, directed against a C-terminal peptide of the erythroid (E) glucose transporter isoform⁸. Initially, we compared the immunolocalization of glucose transporters in cryosections from rat brain, a nonresponsive tissue with regard to insulin-activated glucose transporter, to cryosections of rat heart, an insulin-sensitive tissue which shows the greatest expression of insulin-regulatable glucose transporter². Consistent with previous reports⁹, the erythroid glucose transporter isoform was localized primarily at brain microvessels (Fig. 1a), and no immunofluorescence was detected using monoclonal antibody 1F8 (Fig. 1b). Immunolocalization of glucose transporters with antibody 1F8 in rat heart revealed a very intense immunofluorescence located around capillaries (Fig. 1d). The cardiomyocytes also showed fluorescence after 1F8 incubation, that was not, however, as intense as that observed in capillaries (Fig. 1d). The erythroid glucose transporter isoform detected with antibody B315:32 also exhibited high levels of expression in endothelial cells that exceeded the expression in cardiomyocytes, as determined by indirect immunofluorescence (Fig. 1c). Our previous studies^{10,11} and those of others^{12,13} have shown that both the E and M transporter protein isoforms can be expressed in the same tissue. Additional immunofluorescence studies performed in parallel with a monoclonal antibody against human Von Willebrand factor (Factor VIII antigen), a specific marker of endothelial cells¹⁴, confirmed our analysis that glucose transporters were

TABLE 1 Distribution of insulin-regulatable glucose transporters in endothelial cells from rat heart

	Control	Insulin-treated
Particles per μm^2 of endothelial cell	18.3 ± 2.7	23.6 ± 2.4
Percentage at the luminal side of plasma membrane	6.7 ± 1.8	$47.1 \pm 5.3^*$
Percentage at the basal side of plasma membrane	5.1 ± 1.4	6.8 ± 2.1
Particles per μm of luminal plasma membrane	0.5 ± 0.1	$3.1 \pm 0.4^*$
Particles per μm of basolateral plasma membrane	0.4 ± 0.1	0.5 ± 0.1

Values are means $\pm$ s.e. of 27–29 images representative from six independent experiments (21 μm^2 cell surface area and 70 μm membrane length were evaluated). Data were calculated according to ref. 31 either as the number of gold particles per unit of surface area or as a number of gold particles per unit of plasma membrane length. Key: *, value significantly different from that of the control group, at $P < 0.05$, when analysed by Student's *t* test.

specifically localized in these cells (data not shown). Immunofluorescent analysis was also performed in liver, a tissue which expresses a different glucose transporter isoform^{15,16}, and as expected, no signal was detected with either 1F8 or B315:32 monoclonal antibodies (data not shown).

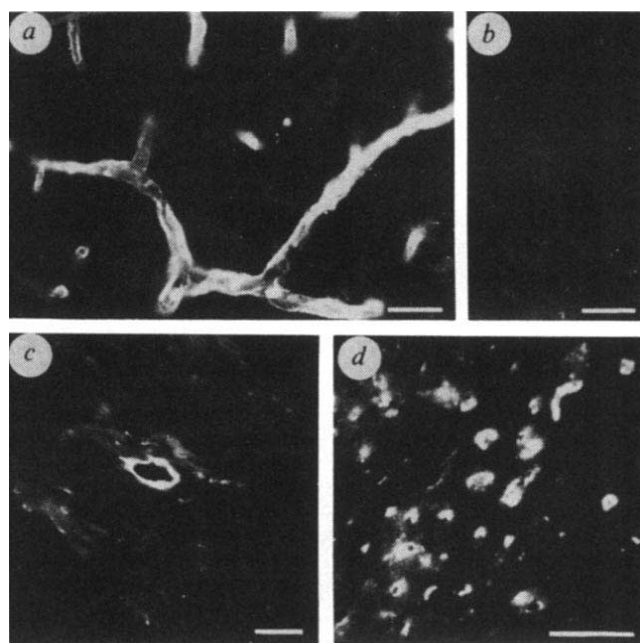


FIG. 1 Differential expression of glucose transporter isoforms in endothelial cells from rat brain and heart. Immunofluorescence in rat cerebellar cortex from brain cryosections after incubation with antibody B315:32 (a) or 1F8 (b). Immunofluorescence of heart sections after incubation with B315:32 (c) or 1F8 (d). Scale bars, 100 μm .

METHODS. Adult male Wistar rats weighing 180–200 g were used. Brains and hearts were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After cryoprotection and freezing, cryostat sections (8–10 μm) were processed for immunofluorescence as described²⁹. Sections were incubated with the primary antibody in 0.1 M glycine, 1% bovine serum albumin, 5% normal goat serum and 0.1% Triton-X-100 in phosphate-buffered saline (pH 7.4) for 2 h at room temperature. The secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:50), was incubated for 1 h at room temperature. Primary antibodies and dilutions were: protein A affinity-purified monoclonal 1F8 (ref. 1) (1:50) and monoclonal B315:32 (ref. 8) directed against a C-terminal peptide of the human Hep G2 glucose transporter (1:50). Antibodies 1F8 and B315:32 do not show any crossreactivity as judged by western blot analysis (data not shown). Results are representative from 10 separate experiments.

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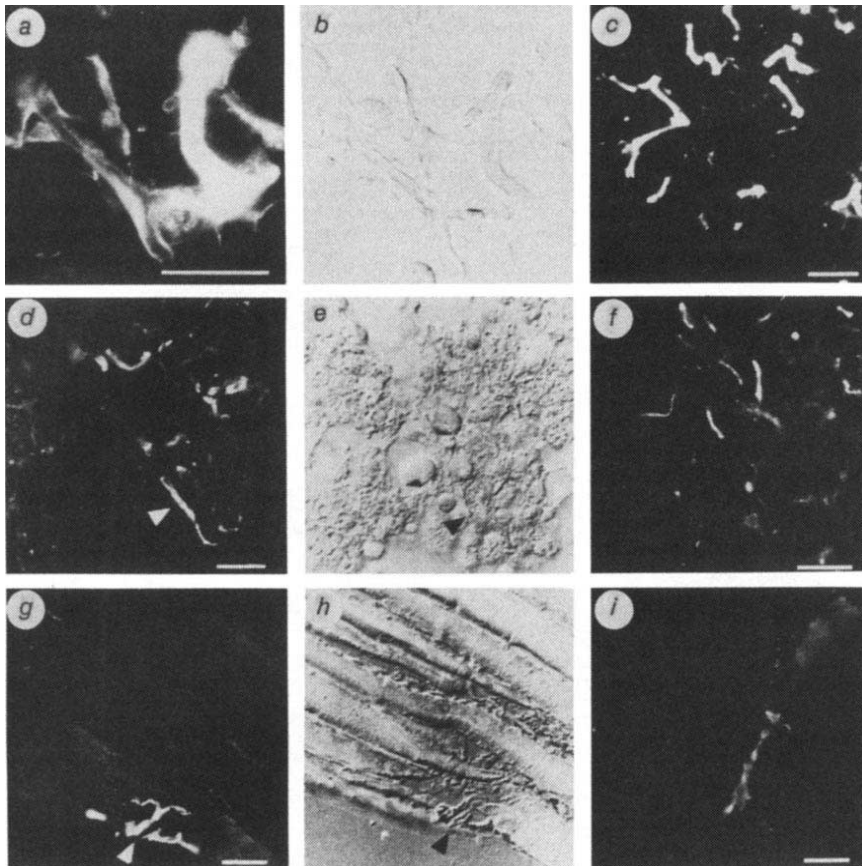


FIG. 2 Endothelial cells from insulin-sensitive tissues highly express the insulin-regulatable glucose transporter. Immunofluorescence in 4% paraformaldehyde fixed tissues was performed as described in Fig. 1. Cryosections of white adipose tissue (a–c) brown adipose tissue (d–f) and skeletal muscle (g–i) were incubated with antibody 1F8 (a, d, g) or antibody B315:32 (c, f, i). Nomarski differential interference microscopy images of the corresponding fields a, d and g are also shown in b, e and h. Arrows in d, e, g and h indicate several representative capillaries visualized both by immunofluorescence and Nomarski differential interference microscopy. Results are representative from four separate experiments. Scale bars, 100 μ m.

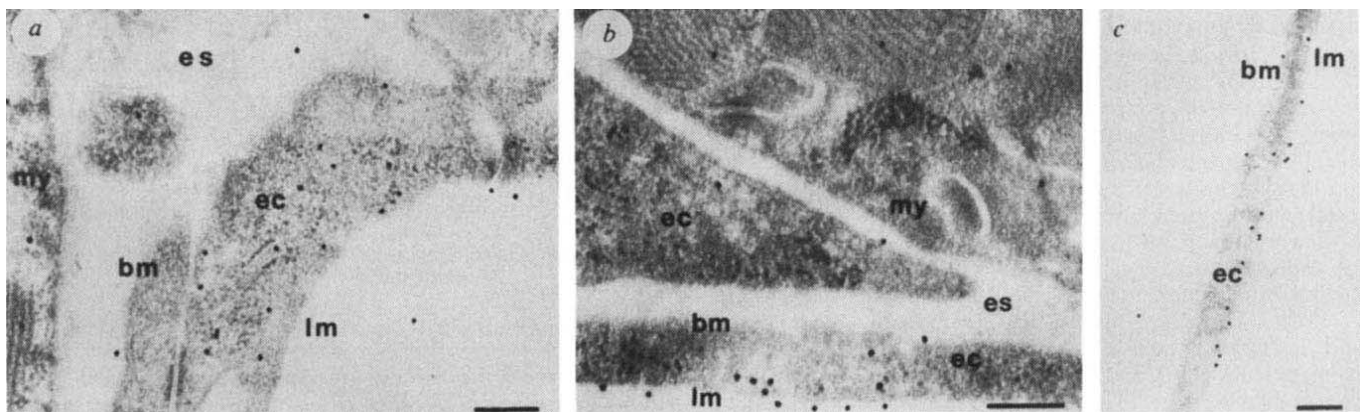


FIG. 3 Insulin causes a rapid increase in the expression of the insulin-regulatable glucose transporter in the plasma membrane from endothelial cells. Ultrathin sections of hearts from control rats (a) or insulin-treated rats (b and c) were immunolabelled with 20-nm protein A-gold particles for the demonstration of insulin-regulatable glucose transporters. The labelling density on the various parts of the cell is shown in Table 1. Key: ec, endothelial cell; es, extracellular space; my, myocyte; bm, basal plasma membrane (facing the myocyte) of endothelial cell; lm, luminal plasma membrane (facing the capillary lumen) of endothelial cell. Scale bars, 250 nm. METHODS. Hearts from 180–200 g male Wistar rats, under basal conditions

or after 15 min intravenous insulin injection (1 unit per kg body weight), were perfused with 2% paraformaldehyde, 0.1% glutaraldehyde, and embedded with Lowicryl K4M as described³⁰. Ultrathin sections were incubated with monoclonal antibody 1F8 (1:15) for 2 h, washed, incubated with rabbit anti-mouse IgG (1:75) for 1 h and exposed to gold-conjugate protein A for 1 h. Several ultrathin sections were processed in parallel with a monoclonal antibody against alcohol dehydrogenase from *Drosophila* and no labelling of any structure was detected, indicating the specificity of the signal caused by antibody 1F8. Results are representative from six independent experiments.

We addressed the question of whether this high and selective expression of the muscle/fat glucose transporter in microvascular endothelium from rat heart was a common property of heart and skeletal muscle, brown and white adipose tissue. These are the major tissues in which insulin stimulates glucose uptake in an acute and dramatic manner, and which abundantly express the muscle/fat glucose transporter isoform^{1–6}. We performed immunocytochemical analyses to localize the M and E

transporter isoforms, as well as the Factor VIII antigen. As was seen for the heart, capillaries exhibited a much stronger immunofluorescence with 1F8 as compared to the neighbouring muscle and fat cells in all tissues subjected to study (Fig. 2a, d, g). Nomarski differential interference microscopy images of the same fields showed that the strongest immunostaining corresponded to the capillary endothelium (Fig. 2b, e, h). Again, the pattern of distribution of the M transporter isoform was similar

to the distribution of Factor VIII antigen (data not shown). Also in keeping with data obtained in heart, antibody B315:32 labelled more strongly capillaries than muscle or fat cells in all tissues investigated (Fig. 2c, f, i).

In fat cells, and, most probably, in muscle, insulin causes the rapid translocation of glucose transporter from an intracellular site to the plasma membrane^{17,18}. To determine if the muscle/fat glucose transporters of capillary endothelium exhibit this type of hormonal regulation, we performed immunolocalization experiments under the electron microscope with the antibody 1F8 in ultra-thin sections from heart muscle using the immunogold technique. Antibody 1F8 recognized a protein more abundantly expressed in endothelial cells than in cardiomyocytes (Fig. 3a, b). Morphometric quantification of data obtained under basal conditions, indicated that the majority of label was located in an intracellular compartment of the endothelial cells, just 12% of label being associated with the plasma membrane (6.7% at the luminal side of plasma membrane and 5.1% at the basal side of plasma membrane) (Fig. 3a, Table 1). The tissue fixation conditions do not allow us to specify which intracellular compartment contains the M glucose transporter isotype, because they do not completely preserve membrane structures. In parallel, heart muscles from insulin-treated rats were also examined. After 15 min of intravenous insulin administration (1 unit per kg body weight), the number of insulin-regulatable glucose transporters located at the luminal side of the plasma membrane in endothelial cells exhibited a 6-fold increase, whereas no change was detected in the total number of glucose transporters per unit of surface area or in the number of transporters located at the basal side of the plasma membrane (Fig. 3b, c; Table 1). Thus, as a consequence of insulin administration, almost 50% of all insulin-regulatable glucose transporters were localized at the luminal side of plasma membrane in endothelial cells (Table 1).

Previous studies of vascular cells isolated from several sources have indicated that these cells possess insulin receptors^{19,20}, and insulin is known to cause a variety of biological effects²¹ including a stimulation of glucose uptake²². However, there are no previous reports of a rapid, insulin-dependent translocation of glucose transporters in endothelial cells. Other studies have indicated that microvessels vectorially transport insulin from the blood to the target tissue²³, and it has been suggested that this step may be rate limiting for insulin action²⁴. Taken together, our data and that published by others strongly favour the concept that endothelial cells regulate insulin and glucose availability and, therefore glucose use in insulin-sensitive tissues. This would explain the correlation found between glucose tolerance and capillary supply to muscle^{25,26}, or between capillary density and fasting plasma glucose²⁷. In addition, our results permit us to propose that peripheral insulin resistance can also be due to specific alterations located at the level of endothelial cells from insulin-sensitive tissues. There is also experimental support for this notion in that capillaries from the fat of diabetic animals show blunted insulin responsiveness *in vitro*²⁸.

In summary, our results demonstrate the high and selective expression of the muscle/fat glucose transporter in endothelial cells from the major insulin target tissues. The localization of the insulin-regulatable glucose transporter at the luminal side of plasma membrane is rapidly and greatly increased after *in vivo* insulin administration, which is in keeping with a hormone-modulated vectorially-defined glucose transporter translocation. Our results suggest a critical role of capillary endothelium from insulin-sensitive tissues in the regulation of glucose availability to fat or muscle cells. That supposes a change in our view of the mechanisms by which glucose metabolism is modified in response to hormones in mammalian tissues. □

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Competition for antigen presentation in living cells involves exchange of peptides bound by class II MHC molecules

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T CELLS recognize foreign proteins as peptides bound to self molecules encoded by the major histocompatibility complex (MHC)¹. The kinetics of interaction between purified class II MHC molecules and peptides is unusual, in that the rate of association is very slow, but once formed, the complexes are extremely stable². This raises the question of how the antigen-presenting cell provides a sufficient number of free MHC binding sites to ensure T cell immunity. We present results suggesting that an exchange of peptide in MHC binding sites may take place under physiological conditions.

Our experiments look at the presentation of peptides from Hen Egg Lysozyme (HEL). We use two T cell hybridomas, one (2C8.4) specific for HEL peptide 112-129 presented by I-A^k molecules³, the other (1H11.3), specific for HEL peptide 105-120 presented by I-E^d molecules⁴. The IL-2 response of these hybridomas provide an assay for the presence of these peptide-MHC complexes on the surface of glutaraldehyde-fixed LK 35.2 (I-A^{k,d} and I-E^{k,d}-expressing) cells. To investigate whether MHC-bound peptides can be exchanged, we test the effect of incubating LK35.2 cells, either live or already fixed, with both antigenic peptide and competitor peptides (the I-A^k-binding HEL peptide⁵ 46-61 or 47-61, or the I-E^d-binding HEL peptide⁴

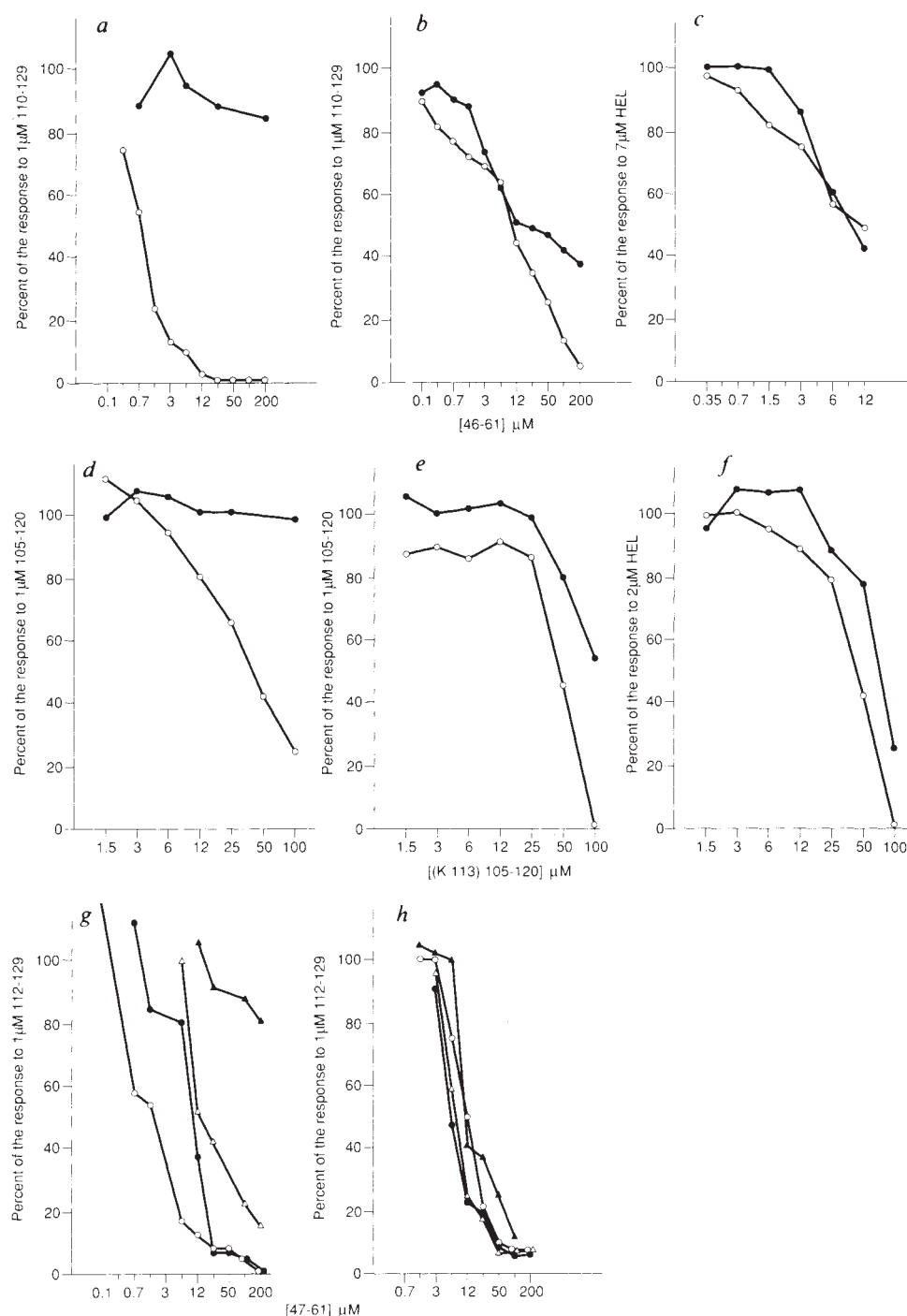
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FIG. 1 Competition for antigen presentation in glutaraldehyde-fixed (*a, d, g*) and living (*b, c, e, f, h*) APC. Glutaraldehyde-fixed (5×10^4 per well) and living (2.5×10^4 per well) LK 35.2 cells were incubated with $1 \mu\text{M}$ of antigenic peptide 110-129 (*a, b*) or 105-120 (*d, e*), or with $7 \mu\text{M}$ of HEL (*c, f*), and a dose-range of competitor peptide 46-61 (*a, b, c*) or (K113) 105-120 (*d, e, f*). Competitor peptides were added simultaneously with the antigen ($\circ$) or subsequently ($\bullet$) (8 h later in *a, b, c*, and 24 h later in *d, e, f*). In the time-course experiment (*g, h*) the competitor peptide 47-61 was added simultaneously with the antigenic peptide 112-129 ($\circ$), or 2 h ($\bullet$), 4 h (Δ) and 6 h ($\blacktriangle$) later. Incubation was continued for 24 h after addition of the competitor. Living APC were then glutaraldehyde-fixed, all cells were washed three times, and 5×10^4 cells per well of the T hybridomas 2C8.4 (*a, b, c, g, h*) or 1H11.3 (*d, e, f*) were added. After 24 h antigen-specific T cell growth factor production was determined by [^3H]thymidine incorporation into CTLL cells. Data are presented as percent of the [^3H]thymidine incorporation (arithmetic mean of triplicate cultures) obtained in response to the antigenic peptide or to HEL. Control responses in the absence and presence of antigen were: 513, 15,984 (*a*); 414, 74,593 (*b*); 372, 31,101 (*c*); 768, 195,762 (*d*); 1012, 254,407 (*e*); 1028, 298,690 (*f*); 756, 38,226 (*g*); and 583, 16,585 (*h*).

METHODS. The synthesis, purification and analysis of HEL peptides and the establishment of T cell hybridomas have been described previously^{3,4}. Cultures containing 5×10^4 T hybridoma cells and 2.5×10^4 LK 35.2 (H-2^{K/d}) cells (obtained from the American Type Culture Collection) were set up with or without antigen in 0.2 ml of RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine, $50 \mu\text{M}$ 2-mercaptoethanol, $50 \mu\text{g ml}^{-1}$ gentamicin (Sigma) and 10% fetal calf serum (Gibco). After 24 h of culture, $50 \mu\text{l}$ aliquots of supernatant were assayed for the presence of T cell growth factors by tritiated thymidine incorporation into 10^4 CTLL cells⁵. Competition for antigen presentation was tested on living or fixed LK 35.2 cells. Cells were fixed in 0.025% glutaraldehyde (Fluka) for 30 s (ref. 3). Living (2.5×10^4 per well) or fixed (5×10^4 per well) LK 35.2 cells were incubated with the indicated concentration of competitor (0.1–200 μM) and antigen (1–2 μM) for 24 h at 37 °C. Living LK 35.2 cells



were fixed at the end of the incubation period. The APC were then washed three times and the T cell response assayed as above.

(K113) 105-120. The competitor peptide is added either at the same time as, or several hours after adding antigenic peptide, and the incubation continued in the presence of both peptides for a further 24 h before assessing the T cell response. In the experiments where the competitor peptide is added several hours after the antigenic peptide, effective inhibition of the T cell response should be seen only if displacement of the MHC-bound antigenic peptide occurs.

The results demonstrate that if the LK 35.2 antigen-presenting cells (APC) are fixed with glutaraldehyde before incubation with peptide, efficient competition for MHC-binding sites is seen only when antigenic and competitor peptides are added simultaneously. If the competitor is added 2 or 4 h later the

inhibitory effect is much less, and by 6 h the competitor peptide has practically no effect even at a 200-fold molar excess (Fig. 1*a, d, g*). In living APC, however, competition occurs irrespective of whether the competitor peptide is added together with the antigenic peptide or up to 24 h later (Fig. 1*b, e, h*). Thus in glutaraldehyde-fixed APC it appears that competition for free MHC sites can occur, but once the peptide-MHC complexes are formed they cannot be dissociated, under the conditions tested, by adding a large excess of competitor. In living APC, however, replacement of one peptide by another can take place. Competitor peptides are also able to replace the antigenic determinants derived by the processing of native HEL (Fig. 1*c, f*).

We then examined the temperature requirements for peptide

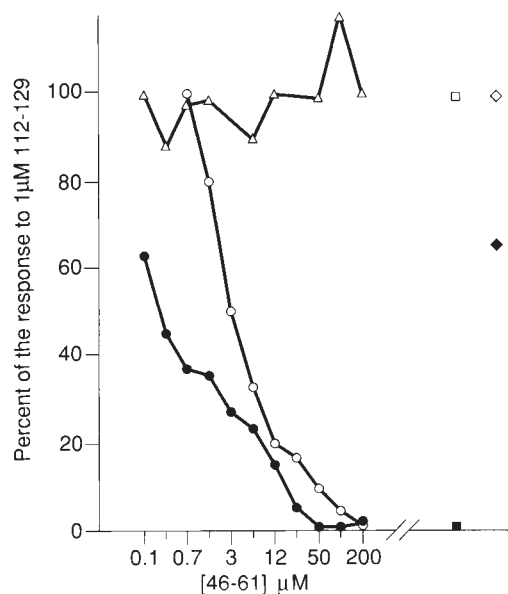


FIG. 2 Temperature dependence of competition for antigen presentation. Living LK 35.2 cells were incubated with 1 μ M peptide 112-129 and with a dose-range of the competitor peptide 46-61. The competitor was added simultaneously with the antigenic peptide ($\square$), or 6 h ($\bullet$) later. Incubation was carried out for a total of 24 h at 37 $^{\circ}$ C. Alternatively, LK 35.2 cells were incubated with the antigenic peptide for 6 h at 37 $^{\circ}$ C, the competitor was then added and the incubation continued at 18 $^{\circ}$ C (Δ). After a total of 24 h incubation APC were glutaraldehyde-fixed and the response of the T hybridoma 2C8.4 was assessed as in Fig. 1. Responses in the absence and presence of 1 μ M peptide 112-129 were 484 and 20,837 c.p.m. respectively. As controls, LK 35.2 cells were cultured with 7 μ M HEL at 37 $^{\circ}$ C ($\square$) and 18 $^{\circ}$ C ($\blacksquare$) or with 1 μ M 112-129 at 37 $^{\circ}$ C ($\diamond$) and 18 $^{\circ}$ C ($\blacklozenge$). After 24 h incubation APC were glutaraldehyde-fixed and the response of the T cell hybridoma 2C8.4 was assessed as in Fig. 1. Responses to HEL and to 112-119 at 37 $^{\circ}$ C were 155,699 and 21,826 c.p.m., respectively.

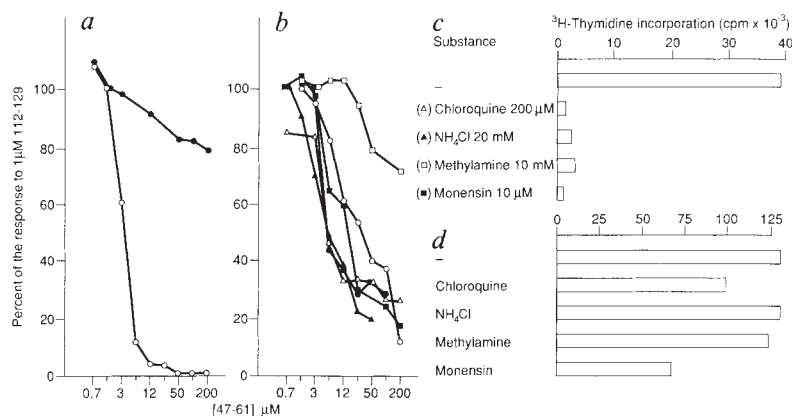


FIG. 3 Effect of lysosomotropic agents on peptide competition. Glutaraldehyde-fixed (*a*) or living (*b*) LK 35.2 cells were incubated with 1 μ M peptide 112-129 and with a dose-range of the competitor peptide 47-61. The competitor was added simultaneously with the antigenic peptide ($\square$) or 6 h later ($\bullet$). The lysosomotropic agents chloroquine (Δ), NH_4Cl ($\blacktriangle$), monensin ($\blacksquare$) at the indicated concentrations were added to living APC 30 min before the competitor peptide, except methylamine ($\square$) which was added 2 h before, according to the time required for maximal increase of endosomal pH (ref. 14), and were left in culture for the entire time course of competition. The experiment was then continued as in Fig. 2. Control responses in the absence or presence of peptide 112-129 were: 396, 14,694 (*a*); and 978, 50,780 (*b*). In the presence of lysosomotropic agents (*b*) control responses to peptide 112-129 were: 15,945 (chloroquine); 21,239 (NH_4Cl); 18,151 (methylamine); 7,903 (monensin). To assess the effect of lysosomotropic agents on antigen processing (*c*), living LK 35.2 cells were incubated with the drugs and 7 μ M HEL for 24 h, then glutaraldehyde-fixed, and their capacity to induce response of the T cell hybridoma 2C8.4 was tested. In addition, the reversibility of drug action was tested (*d*) by incubating LK 35.2 cells with lysosomotropic agents, removing drugs thereafter by washing, and assessing the capacity of APC to process HEL (7 μ M), and present it to hybridoma 2C8.4. Results are expressed as [^3H]thymidine incorporation into 10^4 CTLL induced by 50 μ l aliquots of culture supernatant. Background proliferation of CTLL cells was 978 (*c*) and 3,112 (*d*) c.p.m.

exchange. Living APC were incubated with the antigenic peptide for 6 h at 37 $^{\circ}$ C, and the incubation was continued at either 37 $^{\circ}$ C or 18 $^{\circ}$ C in the presence of competitor peptide. Peptide exchange appears to be completely inhibited at 18 $^{\circ}$ C (Fig. 2) as is processing of native HEL (see legend to Fig. 2). The T cell response to the antigenic peptide 112-129 shows that peptide binding to free MHC sites can still occur at 18 $^{\circ}$ C.

Neutralization of low pH compartments by lysosomotropic agents prevents antigen-processing, for which limited proteolytic cleavage in an intra cytoplasmic, probably endosomal acidic compartment, is considered to be a critical step⁶. To test whether an acidic environment is also important for peptide exchange we investigated the effects of four different lysosomotropic agents. All the lysosomotropic agents used (chloroquine, ammonium chloride, monensin and methylamine) prevented the processing of native HEL almost completely (>95%, Fig. 3c) but only methylamine inhibited peptide exchange (Fig. 3b). It would seem that peptide exchange in the class II binding site

does not require an acidic environment. As most lysosomotropic agents have pleiotropic activities⁷, we assume that the observed effect of methylamine may be independent of its activity on endosomal pH.

Our results do not favour the view that only newly synthesized MHC molecules are available for peptide binding⁸, supporting instead the possibility of a mechanism for peptide exchange that may act during the intracytoplasmic recycling of MHC molecules^{9,10}. The exchange of peptides is temperature dependent and it requires metabolically active APC. Unlike antigen processing, peptide exchange does not require an intracytoplasmic acidic environment, suggesting that these two processes might take place in different cellular compartments. The location of this putative peptide-exchange compartment is not clear. Our results do not rule out the possibility of the cell surface, although the early endosomes, where sorting and recycling of membrane receptors occurs in a mildly acidic environment^{11,12} would seem to be a more attractive candidate given that treatments known

to block endosomal activity (18°C , methylamine¹³) also interfere with peptide exchange. A cellular mechanism that facilitates peptide exchange in MHC binding sites is of physiological importance, as the spontaneous rate of dissociation (T_{50} : 8 h at 37°C for cell-free complexes²) is probably too slow to provide enough binding sites available to new antigens to ensure efficient T cell immunity. □

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Co-association of CD3 ζ with a receptor (CD16) for IgG Fc on human natural killer cells

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NATURAL killer (NK) cells are a subset of lymphocytes that mediate major histocompatibility complex (MHC)-nonrestricted cytotoxicity against tumours and virus-infected cells and secrete numerous cytokines on activation¹. NK cells are distinct from mature T lymphocytes, because they do not rearrange or productively transcribe T-cell receptor α -, β -, γ - or δ -chain genes and do not express the CD3 γ - or δ -subunits^{2–6}. But recent studies indicate that NK cells do express CD3 ζ , co-associated with other membrane proteins⁷. Here we report that CD16, the receptor for the Fc (constant) region of IgG, specifically associates with the CD3 ζ homodimer on the membrane of human NK cells, and that co-transfection with CD3 ζ complementary DNA permits expression of a transmembrane-linked CD16 complex on COS-7 cells. These findings indicate that CD3 ζ can co-associate with

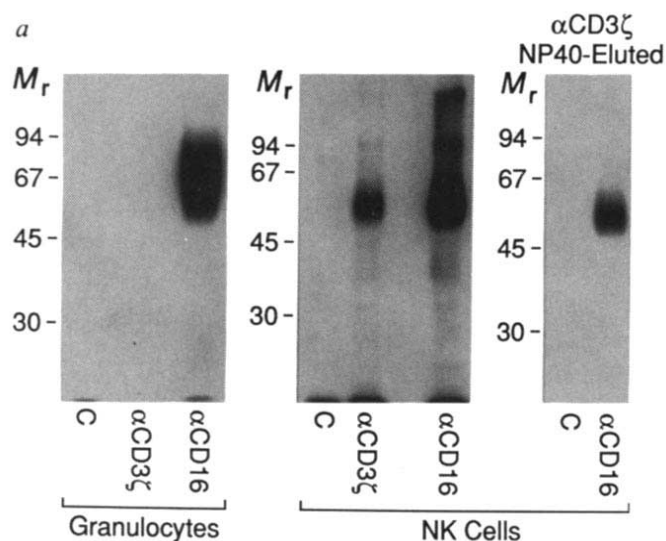
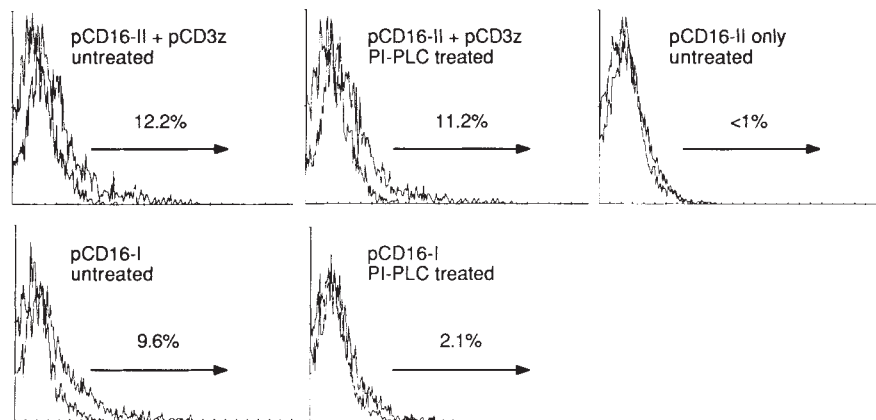


FIG. 1 Co-immunoprecipitation of CD16 and CD3 ζ . *a*, Immunoprecipitation of CD16 and CD3 ζ from ¹²⁵I-membrane-labelled NK cells and granulocytes. *b*, Cleveland peptide map of ¹²⁵I-labelled CD16 and CD3 ζ co-associated proteins on NK cells. *c*, Diagonal SDS-PAGE analysis of anti-CD3 ζ immunoprecipitates from ³⁵S-metabolically-labelled NK cells. *M_r*, relative molecular mass $\times 10^{-3}$.

METHODS. *a*, Viable interleukin-2-dependent NK cell lines (>99% CD3 ϵ ⁺CD56⁺ lymphocytes)²¹ and granulocytes were labelled with ¹²⁵I using lactoperoxidase-glucose oxidase and lysed in 10 mM Tris buffer, 150 mM NaCl, pH 7.2, containing 1% digitonin (Calbiochem), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 20 kU ml⁻¹ aprotinin²⁵. Nuclei were removed by centrifugation at 13,000g for 5 min, and cell lysates were precleared three times with 10 mg packed Pansorbin (Calbiochem-Behring) San Diego, California) coated with saturating amounts of rabbit anti-mouse immunoglobulin serum. Antigens were immunoprecipitated with Pansorbin precoated with control rabbit immunoglobulin (C), rabbit anti-CD3 ζ antibody²⁶ (provided by Dr L. Samelson, NIH), or anti-CD16 antibody (CLB-FcGran1, provided by Drs P. Tetteroo and T. Huizinga, Amsterdam). The CD3 ζ -CD16 complex was immunoprecipitated from digitonin lysates using anti-CD3 ζ antiserum; CD16 antigen was eluted from the immunoadsorbent by incubation for 3 h at 4 °C in 200 μl of 50 mM Tris buffer, 150 mM NaCl, pH 8, containing 1.5% NP-40 and protease inhibitors. The eluted material was re-immunoprecipitated with Pansorbin coated with control immunoglobulin or anti-CD16 monoclonal antibody. Samples were denatured in sample buffer containing 5% 2-mercaptoethanol, and analysed by SDS-PAGE using 10% acrylamide gels^{13,25}. *b*, *M_r* 50–70K bands were cut from the anti-CD3 ζ and anti-CD16 lanes in *a* and were digested for 1 h with 5 and 25 μg of *S. aureus* V8 protease and analysed by SDS-PAGE using 15% acrylamide gels²⁷. *c*, Interleukin-2-dependent NK cell line was metabolically labelled for 18 h in methionine- and cysteine-free RPMI-1640 (Gibco Select Amine Kit, Grand Island, New York) containing 0.2 mCi ml⁻¹ [³⁵S]methionine and cysteine (Tran³⁵S-label, ICN, Irvine, California), 25 mM HEPES buffer (pH 7.4), gentamicin and 5% dialysed FCS. Cells were lysed in 50 mM Tris buffer, 150 mM NaCl, pH 8, containing 1% NP-40, 1 mM PMSF and 20 kU ml⁻¹ aprotinin for 20 min at 4 °C. Nuclei were removed by centrifugation at 13,000g for 5 min, and lysates were immunoprecipitated with control rabbit immunoglobulin or anti-CD3 ζ antibody. Samples were analysed by two-dimensional diagonal SDS-PAGE (first dimension, non-reducing; second dimension, reducing) using 12.5% acrylamide gels²⁵.

FIG. 2 Co-transfection of CD16-II and CD3 ζ . COS-7 cells were transfected with plasmids pCD16-I or pCD16-II only, or pCD16-II and pCD3 ζ , treated with phosphatidylinositol-specific phospholipase C (PI-PLC), stained with phycoerythrin anti-CD16 monoclonal antibody, and analysed by flow cytometry. METHODS. CD16-II was isolated from a λ gt11 library ($\sim 5 \times 10^5$ recombinant phage) constructed using cDNA prepared from poly (A)⁺ RNA of rIL-2-cultured human NK cells²⁸ and subcloned into the CDM8 expression vector²⁹, designated pCD16-II. Human CD3 ζ cDNA was isolated by polymerase chain reaction (PCR) from a Jurkat cDNA CDM8 plasmid library using primers flanking the open reading frame (5' primer GGCAAGCTTTCCCGGCCACCGCTGCCTCAG and 3' primer GCCCTCGAGTGGCGCTGTCAGGTCTGGCC). HindIII and XhoI sites were included in the 5' and 3' primers, respectively, and the CD3 ζ PCR product was digested with HindIII/XhoI and ligated into the CDM8 vector, designated pCD3 ζ . COS-7 cells were transfected with pCD16-I (provided by Dr B. Seed, Boston) or pCD16-II only, or pCD16-II and pCD3 ζ using DEAE-dextran, collected after 48–72 h, and analysed for expression²⁹. Transfectants were incubated for 1 h at 37 °C in the presence or absence of *Bacillus thuringiensis* PI-PLC



(0.1 U ml⁻¹, Immunotech, Marseille, France), washed, and stained with phycoerythrin-conjugated anti-Leu 11c (CD16) or a phycoerythrin-conjugated IgG1 control monoclonal antibody, and analysed by flow cytometry²¹. The x axis represents fluorescence (four decade log scale), and the y axis represents the relative cell number. Histograms from cells stained with control monoclonal antibody (nearest the ordinate) are superimposed.

membrane receptors of diverse cell types and function as a common structure for signal transduction.

Viable NK cells (CD3 ϵ^- , CD16 $^+$, CD56 $^+$ lymphocytes) were labelled with ¹²⁵I, solubilized under conditions to preserve non-covalent associations of membrane protein complexes, and immunoprecipitated with anti-CD16 or anti-CD3 ζ antibodies. Because CD3 ζ does not possess tyrosine residues in the extracellular region⁸, it is not ¹²⁵I-labelled using viable nonpermeabilized cells. But anti-CD3 ζ antibody co-immunoprecipitated an ¹²⁵I-labelled glycoprotein of relative molecular mass 50,000–70,000 (*M_r* 50–70K), identical in mobility to CD16 (Fig. 1a). Identity of this co-associated structure as CD16 was confirmed by elution of the glycoprotein from the anti-CD3 ζ immunoadsorbent with 1.5% Nonidet P-40 detergent and re-immunoprecipitation with anti-CD16 monoclonal antibody (Fig. 1a) and by peptide mapping using *Staphylococcus aureus* protease V8 (Fig. 1b). Although it is possible that CD3 ζ co-associates with other membrane proteins on NK cells, no other CD3 ζ -associated structures were detected under the experimental conditions that we used. Anti-CD3 ζ antibody failed to immunoprecipitate any structures from ¹²⁵I-labelled granulocytes (Fig. 1a), which express the phosphatidylinositol-glycan form of CD16 (refs 9–11), showing that there is no nonspecific cross-reactivity of anti-CD3 ζ antibody with CD16. Expression of CD3 ζ by NK cells was confirmed by immunoprecipitation of the characteristic CD3 ζ disulphide-linked homodimer from NK cells metabolically labelled with [³⁵S]methionine and cysteine (Fig. 1c). Moreover, CD3 ζ messenger RNA in NK cells was detected by northern-blot analysis, but not in granulocytes (data not shown).

Anderson recently reported co-association of CD3 ζ with several different proteins on NK cells, and suggested that these structures could be membrane receptors involved in tumour-cell recognition and cellular activation⁷. CD16 fulfils the criteria of a functional membrane receptor and transduces signals through the phosphatidylinositol pathway¹². Binding of immune complexes or monoclonal antibodies against CD16 to NK cells causes a rapid increase in intracellular Ca²⁺ and inositol 1,4,5-trisphosphate generation, with subsequent transcription of lymphokines and triggering of cell-mediated cytotoxicity^{12,13}. Antigen-induced T-cell activation results in tyrosine phosphorylation of CD3 ζ (refs 14–17). Efficient membrane expression of CD3–T-cell receptor (TCR) complex and signal transduction through the CD3–TCR complex apparently requires expression of CD3 ζ (refs 14–17). Given the role of CD3 ζ in activation through the

TCR complex^{14–17}, it is likely that CD3 ζ is involved in signal transduction through transmembrane-anchored CD16. Although CD16 is undoubtedly an important signal-transducing structure, we have identified a subset of NK cells that do not express CD16 (refs 18 and 19). These NK cells efficiently kill tumour-cell targets, indicating that structures other than CD16 also mediate this function.

Two highly homologous genes encode CD16 (ref. 20). *CD-16-I* encodes a phosphatidylinositol glycan-linked glycoprotein expressed by granulocytes, whereas CD16-II generates a transmembrane-anchored glycoprotein expressed on NK cells and activated macrophages^{9–11,13,20,21}. Whereas cDNA encoding CD16-I is expressed in COS-7 transfectants as phosphatidylinositol glycan-anchored proteins⁹, CD16-II cDNA alone was incapable of membrane expression in COS-7 (Fig. 2a). Co-transfection with CD3 ζ cDNA, however, allowed membrane expression of CD16-II (Fig. 2b). Expression of the CD3 ζ in these COS-7 co-transfectants was confirmed by immunoprecipitation of the homodimer from metabolically labelled cells (data not shown). The CD16 glycoprotein on the membrane of the CD16-II–CD3 ζ COS-7 transfectants was resistant to cleavage with phosphatidylinositol-specific phospholipase C (Fig. 2c). By contrast, CD16 was efficiently cleaved from the surface of CD16-I COS-7 transfectants (Fig. 2d). These results directly correspond with the ability of phospholipase C treatment to cleave CD16 from the surface of granulocytes but not NK cells^{20,21}. Recently, Ravetch and colleagues have reported that co-transfection with the γ -subunit of the rat high-affinity IgE receptor also permits membrane expression of CD16-II on COS-7 cells²². Although it is possible that NK cells express both CD3 ζ and the γ -subunit of the high-affinity IgE receptor, so far there are no serological reagents against the human γ -subunit to examine this possibility.

The requirement for the CD3 ζ homodimer for membrane expression of CD16 in NK cells is analogous to the requirement for the γ -homodimer for expression of the high-affinity IgE Fc receptor present on mast cells and basophils²³. Note that the transmembrane segment of the γ -subunit of the IgE Fc receptor is highly homologous to the CD3 ζ transmembrane segment^{23,24}, indicating that the interaction site is between residues in the transmembrane segments of CD3 ζ and CD16. These results indicate that CD3 ζ or a family of homologous structures can co-associate with membrane receptors involved in signal transduction in several different cell types. □

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A single amino acid in the glycosyl phosphatidylinositol attachment domain determines the membrane topology of Fc γ RIII

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CELL-SURFACE proteins are associated with the lipid bilayer either as membrane-spanning molecules or as glycosyl phosphatidylinositol (GPI)-linked proteins. Proteins destined for GPI anchoring are synthesized as precursors with a hydrophobic C-terminal transmembrane domain, which is removed during the processing of these proteins in the endoplasmic reticulum (ref. 1). We have investigated the structural requirements for GPI anchoring through the study of two closely related proteins which exhibit alternative membrane attachment. The IgG Fc receptor, Fc γ RIII, is GPI-linked on neutrophils (III-1)²⁻⁴ whereas on natural killer (NK) cells and macrophages it is found as a transmembrane-anchored molecule (III-2)^{4,5}, able to mediate antibody-dependent cellular cytotoxicity and phagocytosis⁶. At the primary structural level, the III-1 gene differs from that encoding III-2 by only nine nucleotide substitutions, which result in six amino-acid differences, and the absence of 21 amino acids at the C terminus^{4,5,7}. We have analysed a series of III-1 and III-2 mutants in transient expression assays, and show that Ser 203 in the GPI attachment domain is the dominant residue in determining whether the molecule can be GPI-anchored. As in the case of its murine homologue, Fc γ RII α , surface expression of the III-2 molecule is dependent on co-expression of a second subunit, the γ chain of Fc ϵ RI. Our data also suggest that γ chain can associate with the III-1 precursor, preventing GPI attachment, favouring instead a transmembrane form.

To investigate the primary structural requirements for GPI attachment in Fc γ RIII molecules, chimaeric molecules

were constructed from complementary DNA clones for III-1 and III-2 (ref. 4) (Fig. 1). These chimaeric constructs were designed to test the contribution of the cytoplasmic tail, and the substitutions found in the extracellular domains, on the topology of the molecules. Previous studies have indicated that the length of the intracytoplasmic domain influences the membrane topology of alternatively anchored proteins, like LFA-3 (lymphocyte function associated antigen-3) and N-CAM (neural cell-adhesion molecule)^{8,9} and that substitution of a single amino acid (Asp $\rightarrow$ Val) in the transmembrane region of QaQ7 converted this GPI-anchored molecule to a transmembrane protein¹⁰. We therefore designed additional constructs, altering Asp 222 to Val in III-1 and III-2, and testing the effect of combining this transmembrane mutation with either a short (III-1 like) or long (III-2 like) cytoplasmic domain.

The molecules described in Fig. 1 were introduced into COS cells to evaluate their membrane topology, as these cells have been previously shown to express GPI-anchored proteins efficiently, including Fc γ RIII-1 (ref. 7). Cotransfections with

Structure of gene	Expression efficiency (%)		PIPLC resistance (%)		Membrane form	
	-Y	+Y	-Y	+Y	-Y	+Y
III-1	100	112	5	71	PI	TM
III-2	<1	55	-	92	-	TM
III-1(F)	<1	111	-	125	-	TM
III-2(S)	95	65	<1	78	PI	TM
1-2(K)	92	94	8	N.D.	PI	N.D.
1-2(A)	<1	107	-	90	-	TM
1-2(P)	<1	74	-	105	-	TM
2-1(K)	<1	65	-	89	-	TM
2-1(A)	87	65	<1	84	PI	TM
2-1(P)	101	104	2	N.D.	PI	N.D.
III-1(V)	73	71	<1	N.D.	PI	N.D.
III-1(V-S)	4	5	<1	N.D.	PI	N.D.
III-2(V)	4	34	107	N.D.	TM	N.D.
III-2(V-S)	4	7	91	N.D.	TM	N.D.

FIG. 1 Expression efficiency and membrane topology of Fc γ RIII genes and mutants in COS cells. The expression efficiency and phosphatidylinositol-specific phospholipase C (PIPLC) resistance for each molecule was determined by monoclonal antibody 3G8 (ref. 22) binding in a rosetting assay as described in the legend to Fig. 2. The schematic structure of each molecule is shown with circles indicating the immunoglobulin-like extracellular domains, the wavy line representing the transmembrane domain, and the amino acid differences noted. PI, GPI-anchored form; TM, transmembrane form; ND, not done; —, not expressed in the absence of γ .

METHODS. The coding sequences of Fc γ RIII-1 (NA-2) and Fc γ RIII-2 cDNAs were inserted into the EcoRI site of pCEXV-3 (refs 4, 11). Chimaeric complementary DNAs were constructed by ligating appropriate fragments from these III-1 and III-2 cDNAs, before insertion into the expression vector. Single amino-acid substitutions were constructed by oligonucleotide directed mutagenesis on single-stranded templates. III-1(V), III-1(V-S) were constructed from the NA-1 allele of Fc γ RIII-1. All constructs were confirmed by DNA-sequence analysis; purity of preparation was confirmed by polymerase chain reaction. The mouse Fc ϵ RI γ subunit was expressed from a cDNA cloned in pCEXV-3. Mixing of this construct with Fc γ RIII constructs (1:1) was performed before DNA transfections as described in Fig. 2. Transfection of γ -subunit alone resulted in no positive cells. Expression efficiency (Eff) was calculated by the following relationship: Eff = No. 3G8 positive cells for mutant/No. 3G8 positive cells for III-1 thereby normalizing each experiment to a standard value for III-1 expression in COS cells. PIPLC resistance (Res) was determined by the relationship: Res = No. 3G8 positive cells + PIPLC/No. 3G8 positive cells - PIPLC. A minimum of 1,000 positive cells were counted per experiment. Data were calculated as the mean values derived from two to four experiments. FACS analysis after staining with 3G8, of III-1, III-2 + γ , III-1(F) + γ and III-2(S) expression, and PIPLC resistance, resulted in values comparable to those obtained by rosette analysis.

the Fc ϵ RI γ chain were included because Fc γ RII α (ref. 11), the murine homologue of Fc γ RIII-2 (ref. 4), is not efficiently expressed by COS cells¹² unless this subunit is also present¹³. Transient expression assays were also performed with L cells, as these cells are unable to express GPtdIns-anchored molecules¹⁴, thereby providing an additional indication of topology.

Transfection experiments using cloned III-1 and III-2 genes, the murine homologue of III-2, Fc γ RII α , and a more distantly related, low-affinity IgG Fc receptor, Fc γ RII β (ref. 11), are shown in Fig. 2. The molecules are cloned in an identical vector, yet the number of COS cells expressing III-2 or Fc γ RII α at the cell surface is less than 1% of that seen after transfection with III-1, or the transmembrane molecule Fc γ RII β . Co-expression of the γ subunit of Fc ϵ RI increases the expression of III-2 by 50–100-fold, as seen in Figs 1 and 2. The sensitivity to phosphatidylinositol-specific phospholipase C (PIPLC in Figs 1 and 3) confirms that III-1 is GPtdIns-anchored to the membrane, whereas the resistance of III-2 to that enzyme indicates a transmembrane topology, as was demonstrated for III-2 on NK cells⁴. A role for γ chain in the cell-surface expression of III-2 is consistent with the observation that γ chain expression (as judged by northern blot analysis) is found on NK cells and macrophages that express III-2 but not Fc ϵ RI (data not shown).

Gamma chain-dependent III-1 expression is also observed on transfecting L cells with the III-1 gene (Fig. 2), indicating that the III-1 molecule can be expressed in the absence of a GPtdIns anchor. The III-1 expressed in this manner is PIPLC-resistant (data not shown), suggesting that it is in a transmembrane and probably γ -associated form. Further investigation showed that even in COS cells, which are capable of GPtdIns

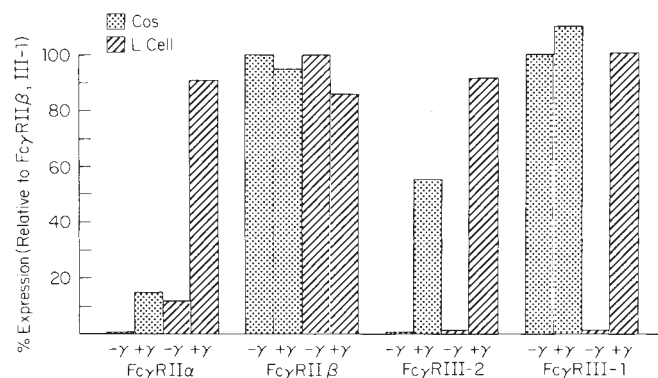


FIG. 2 Effect of the Fc ϵ RI γ subunit on expression of Fc γ Rs in COS and L cells. The results of transfecting either COS or L cells with Fc γ R genes in the presence or absence of Fc ϵ RI γ subunit are displayed. Expression of murine Fc γ RII α and Fc γ RII β were calculated relative to Fc γ RII β whereas expression of human III-1 and III-2 are presented relative to III-1 expression. METHODS. Transfection of L cells, and the immune complex rosetting assay for mouse Fc γ II α and mouse Fc γ II β were described previously^{11,12}. COS cells were plated 24 h before transfection at 2.5×10^5 cells per 60-mm Petri dish. The next day, cells were incubated with serum-free α -MEM (minimal essential medium) containing DEAE-dextran ($150 \mu\text{g ml}^{-1}$), and chloroquine ($100 \mu\text{M}$) with the plasmid DNA at $30\text{--}60 \mu\text{g ml}^{-1}$. After a 16 h exposure to DNA, the cells were shocked with Tris-buffered saline (TBS) containing 20% glycerol for 3 min, washed with TBS and fed with α -MEM supplemented with 10% FCS. A rosetting assay for human Fc γ RIII was performed two days after transfection. The cells were incubated with $0.5\text{--}1.0 \mu\text{g}$ monoclonal antibody 3G8 per for 30 min at 4°C and washed with α -MEM containing 2% FCS and 0.05% NaN₃. Sheep erythrocytes coated with goat anti-mouse IgG were then added to each plate, incubated for 30 min at 4°C and washed extensively with PBS buffer. Percentages of 3G8-positive rosetting cells per 2.5×10^5 cells were as follows: 13–15% (mouse Fc γ RII β in COS cells); 4–8% (mouse Fc γ RII β in L cells); 3–13% (human Fc γ RIII-1 in COS cells); 0.9–1.6% (human Fc γ RIII-1 with the γ subunit in L cells).

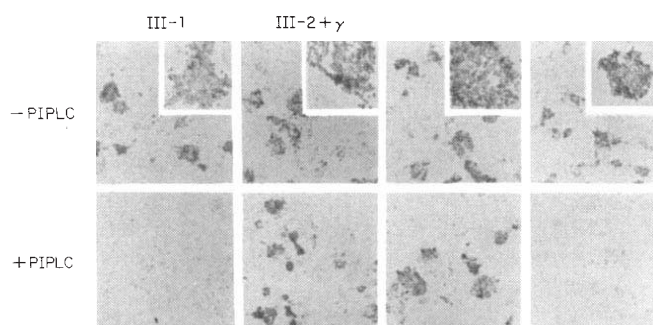


FIG. 3 GPtdIns and transmembrane anchoring of Fc γ RIII in COS cells. The indicated molecules (see Fig. 1) were introduced into COS cells by transfection. Surface expression of Fc γ RIII molecules was determined by monoclonal antibody binding, detected in a rosetting assay. Sensitivity to phosphatidylinositol-specific phospholipase C (PIPLC) is shown for each representative molecule. Inset panels show a higher magnification of an individual rosetting cell.

METHODS. After transfection (48 h), the cells were incubated for 60 min at 37°C , in the absence or presence of PIPLC (1:100 dilution of a preparation purified from *Bacillus thuringiensis* activity 300–400 units per ml) in α -MEM containing 1% BSA. The cells were washed twice before testing for 3G8 binding in a rosetting assay (see Fig. 2), fixed with 0.25% glutaraldehyde and photographed.

processing, III-1 is PIPLC-resistant when expressed in the presence of γ chain (Figs 1 and 4). As γ chain probably interacts with the transmembrane domain (see below), and this domain is cleaved from the III-1 molecule on processing to the GPtdIns-linked form, we interpret these results to mean that association with γ chain can interfere with the GPtdIns-processing that would otherwise occur in COS cells.

Transient expression and phosphatidylinositol-specific phospholipase C resistance allow us to distinguish between an Fc γ RIII molecule that can be GPtdIns-linked in COS cells and one that is expressed in a transmembrane configuration. We evaluated the primary structural requirements for GPtdIns-linkage by testing the Fc γ RIII mutants described in Fig. 1. Exchanging the 25-residue cytoplasmic domain of III-2 for the 4-residue domain of III-1 (2-1(K) in Fig. 1) did not convert III-2 to a GPtdIns-anchored molecule. If exchange of the C-terminal end of the molecule included position 203, however, the ability to form a GPtdIns anchor segregated with the C-terminal end (1-2(A) and 2-1(A)). To confirm that residue 203 is the critical determinant of this alternative anchoring, we made constructs in which only this residue was exchanged. III-2(S) has a Ser instead of a Phe at position 203, with all other positions identical to III-2, and is expressed as a GPtdIns-linked molecule (in the absence of γ chain). Conversely, III-1(F), which is identical to III-1 except for Phe 203, can now be expressed only in the presence of γ chain. Similar results have been obtained independently¹⁵.

Mutation of Asp 222 to Val in the transmembrane domain of III-1 (III-1(V)) did not affect GPtdIns attachment, nor did adding a long cytoplasmic tail to this mutant (III-1(V-L)). The resulting molecule was still GPtdIns-anchored, but demonstrated a 20-fold reduction in its efficiency of expression. The analogous mutants of III-2 (III-2(V) and III-2(V-S)) showed some independence from γ chain and a lower level of γ chain enhancement. It is likely that γ -chain interaction with III-2 is dependent both on the charged amino acid in the transmembrane domain (also found in III-1) and the longer cytoplasmic tail. Homology between the α subunit of Fc ϵ RI and Fc γ RIII in their transmembrane domains^{16,17} supports the view that this subunit functions through its interactions with these membrane-spanning domains^{18,19}. Significant homology is also observed with the zeta chain of the T-cell receptor-CD3 complex²⁰. We found that human zeta chain was able to substitute for γ chain

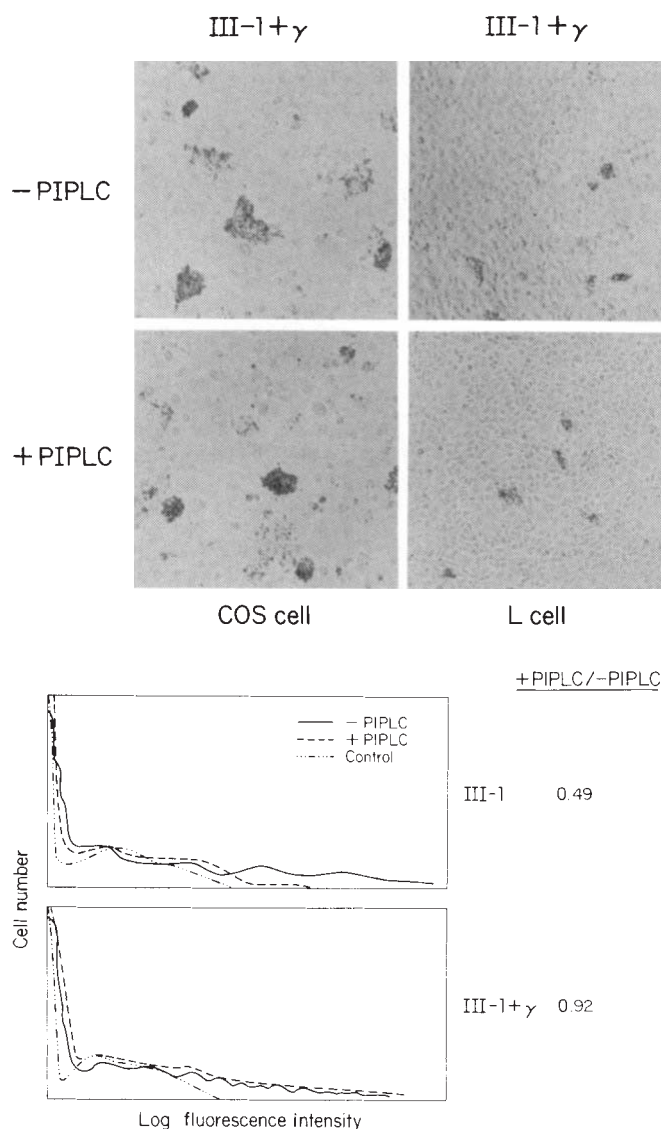


FIG. 4 Transmembrane anchoring of Fc γ RIII-1 in COS and L cells in the presence of γ subunit. Top panel, Transient expression of III-1 on the surface of COS and L cells was detected by a rosette assay (see Fig. 2). PIPLC treatment was performed as described in the legend to Fig. 3. Lower panel, Results obtained for the COS transfectants using FACS analysis of 3G8 binding. PIPLC-resistance was determined from the mean fluorescence intensity obtained in the presence or absence of PIPLC for III-1 and III-1+ γ . Control fluorescence was determined using a nonbinding antibody Xb3.

may associate with γ chain in both COS and L cells to result in a surface-expressed, transmembrane-anchored molecule. This result indicates that the presence of molecules like the γ chain may influence the membrane topology assumed by a protein isoform and represent an alternative point of regulation for those molecules. □

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A mutant protein kinase C that can transform fibroblasts

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EXPRESSION of normal protein kinase C (PKC) isoenzymes in fibroblasts has been shown to alter growth regulation but has failed to induce complete transformation of the recipient cells^{1,2}. Here we report on a murine ultraviolet-induced fibrosarcoma cell line which has an unusual PKC subcellular distribution with 87% of the PKC activity associated with the membrane. We have cloned and sequenced the α -PKC complementary DNA from ultraviolet-induced-fibrosarcoma cells and from mouse Balb/c brain and found four point mutations in the fibrosarcoma PKC, of which three are in the highly conserved regulatory domain and one is in the conserved region of the catalytic domain. Expression of this mutant α -PKC gene in normal Balb/c 3T3 fibroblasts results in a fibrosarcoma-like PKC membrane localization and in cell transformation, as judged by their formation of dense foci, anchorage-independent growth and ability to induce solid tumours when inoculated into nude mice. By contrast, transfectants expressing the normal α -PKC cDNA do not display a morphology typical of malignant transformed cells and fail to induce tumours *in vivo*. These findings demonstrate that point mutations in the primary structure of PKC modulate enzyme function and are responsible for inducing oncogenicity.

Protein kinase C is the primary receptor for the class of tumour-promoting agents known as phorbol esters, and much effort has been directed towards interpreting the role of PKC in the process of tumour growth and development³⁻⁵. Overexpression of normal PKC species in various fibroblasts results

in enhancing surface expression of III-2. However, murine zeta was unable to enhance surface expression of Fc γ RIII α or its human homologue III-2 (data not shown).

Examination of protein sequences that show GPtdIns anchoring reveal several common features¹: (1) GPtdIns attachment occurring at Ser, Cys, Asp, Asn and Gly and not at amino acids that are strongly hydrophobic or bear bulky side chains²¹, in a domain 8-12 amino acids to the NH₂-C terminus of a hydrophobic domain; (2) a weakly hydrophobic, C-terminal transmembrane domain, and (3) a short or absent intracytoplasmic domain. The results obtained from Fc γ RIII indicate an absolute requirement for short-side-chain amino acids, like Ser 203 in the GPtdIns attachment domain which is dominant, although changes in the transmembrane domain or length of the intracytoplasmic domain may effect the overall efficiency of this process. Our results also indicate that the γ chain of Fc ϵ RI interacts with the transmembrane domain of Fc γ RIII. As a consequence of this interaction the membrane-anchored precursor of III-1

TABLE 1 [3 H]PDBu binding and PKC specific activities in UV25 PKC transfectant cell lines

Cell line	[3 H]PDBu binding (pmol per 10^6 cells)	Fold-increase*	Specific PKC activity (pmol min $^{-1}$ mg $^{-1}$ protein)	
			Triton X-100-solubilized particulate fractions	DEAE-purified cytosolic fractions
Balb c/3T3	1.3	1.0	350	1,320
Neo/3T3	2.1	1.6	540	2,240
α -PKC/3T3	13.2	10	4,000	12,200
UV2240	1.0	0.7	800	90
UV15/3T3	3.9	3	4,700	700
UV20/3T3	3.2	2.5	3,800	570
UV25/3T3	13.1	10	11,500	2,800
B-UV25/3T3	13.0	10	3,800	12,100

[3 H]Phorbol-dibutyrate (PDBu) binding was performed on confluent monolayers of cells¹⁰.

* Expressed as the mean of the fold-increase relative to Balb c/3T3. Values observed within individual experiments are $P < 0.5$. Partially DEAE-purified cytosolic fractions and Triton X-100-solubilized particulate fractions were assayed for PKC activity as before¹⁰.

Values are the means of three separate determinations.

All cells were grown in Dulbecco's modified essential medium (DMEM, Gibco), supplemented with 10% fetal calf serum (Gibco), 2 mM fresh glutamine, 100 U per ml penicillin and 100 μ g ml $^{-1}$ streptomycin, in a 37 °C, 5% CO $_2$ humidified environment. Balb c/3T3 cells were not allowed to grow to post-confluence. Plasmids were constructed essentially according to standard procedures²³. The 3,500-base pair-EcoRI fragments containing the cDNAs for UV25 PKC, back-mutated UV25 PKC and the mouse α -PKC were ligated into EcoRI-digested pLTR with the multiple cloning site upstream of the 5' end of the cDNAs. Transfection was as described¹⁵. Briefly, $3-4 \times 10^5$ Balb c/3T3 cells were plated in 10-cm diameter dishes: 12-24 h later they were exposed to a calcium phosphate-DNA precipitate prepared by adding 0.5 ml of a 250 mM calcium-plasmid DNA (DNA concentration, 40 μ g ml $^{-1}$) solution dropwise, while bubbling air, to 0.5 ml of 2 $\times$ HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, pH 7.05, 1.5 mM sodium phosphate, 10 mM KCl and 12 mM dextran) and incubating for 30-45 min at room temperature. Cotransfection was done with a 10-fold excess of the PKC plasmids over the selectable plasmid PSV-NEO (ref. 24). The precipitate was removed 10-18 h later, and the cells washed twice with growth medium. Cells were allowed to recover for 24 h before being trypsinized and split at a ratio of 1:7, after which they were selected by feeding with growth medium containing 300 μ g ml $^{-1}$ of G418 antibiotic (Gibco). Colonies of G418 r cells were apparent after 8-10 days and were subsequently picked after 14-17 days for expansion. Oligonucleotide-directed mutagenesis: Oligonucleotides were synthesized on an Applied Biosystem oligonucleotide synthesizer (Wayne Brown): 30-mer from nucleotide positions 470-500 (covering point mutation 1,2), 29-mer from nucleotides 871-900 (covering point mutation 3) and 22-mer from nucleotides 1,170-1,191 (covering the fourth mutation). The back mutation led to the replacement of Val 106 by Ile, Gly 111 by Ser, Gln 240 by Leu and Leu 339 by Phe, thus converting the UV-25 PKC mutant gene into the wild-type α -PKC gene. An EcoRI fragment of pLTR 25 PKC was cloned into the EcoRI site of M13 and mutagenized (according to standard procedures²⁵, using the Amersham oligonucleotide-directed mutagenesis *in vitro* system). The identity of all point mutations was confirmed by DNA sequencing. The M13 plasmid bearing the back-mutated UV25 cDNA was cleaved with EcoRI, which liberates the full length cDNA. This back-mutated UV25 insert was isolated and ligated into the EcoRI site of pLTR expression vector.

chelator (particulate/soluble ratio of 0.3-0.5)⁹. By contrast, transformed fibroblasts usually show reversed PKC distribution, with a particulate/soluble ratio between 1 and 2 (ref. 9). We have investigated the PKC localization in four highly tumorigenic ultraviolet-induced fibrosarcoma cell lines¹¹. These cells show a PKC distribution pattern similar to the transformed cells, but with more dramatic variation (particulate/soluble ratio of 7). In addition, there is a marked decrease in specific and total PKC activity which correlates with a decreased binding of phorbol esters (Table 1).

These results are indicative of a defect in the PKC of ultraviolet-induced fibrosarcoma cells. Therefore, from the UV2240 cell line we cloned the cDNA for α -PKC shown to be the predominant isoform in this cell line by hydroxyapatite chromatography (data not shown). For comparison we also cloned the α -PKC cDNA from mouse Balb/c brain. The UV2240 and Balb/c brain cDNA libraries were screened under high stringency conditions with a cDNA probe containing the complete open reading frame of the bovine α -PKC (λ -bPKC 306; ref. 12). Isolated positive clones were confirmed by Southern

hybridization and nucleotide sequencing (Fig. 1a). Sequence analysis of UV2240 and mouse α -PKC clones revealed the presence of a complete open reading frame (672 amino acids) with 98% homology to bovine and rabbit α -PKC cDNAs. In the 5' untranslated sequence of the UV2240 PKC cDNA, two in-frame termination codons were located in positions 63 and 92 nucleotides upstream of the potential initiation codon. In mouse α -PKC, a methionine presumptive start codon was located at a position comparable to one in the UV2240 PKC, but no stop codon could be found in the upstream region. Alignment of nucleotides and the deduced amino-acid sequences of the UV2240 cDNA with both Balb/c brain and murine 3T3 cDNA¹³ clones revealed four nucleotide substitutions resulting in mutation of four amino acids. Three of the nucleotide substitutions occurred in the regulatory domain of the enzyme, changing Ile 106 to Val, Ser 111 to Gly, and Leu 240 to Gln. The other nucleotide substitution is in the highly conserved catalytic domain, where Phe 339 is altered to Leu. The amino acids Ser 111, Leu 240 and Phe 339 are highly conserved in the PKC isoenzyme sequences known so far (including mouse¹³, bovine^{12,14}, rabbit¹⁵ and human¹⁴), but Ile 106 is substituted by Leu in PKC- γ isoenzymes^{13,14} (Fig. 1b).

To assess the transforming properties of the mutant PKC gene (clone UV25 from UV2240), we expressed it in Balb c/3T3 fibroblasts and investigated the ability of the transfectants to form foci, display anchorage-independent growth and tumorigenicity in nude mice, all properties that correlate well with the malignant phenotype. The UV25 and the mouse α -PKC cDNAs were introduced into the pLTR expression vector. (Transcription is controlled by Harvey sarcoma virus promoter; Fig. 1c.) These two subclones were co-transfected with PSV-NEO (containing the G418 antibiotic-resistance gene) into Balb c/3T3 cells by Ca $^{2+}$ -phosphate precipitation¹⁶ and transfected cells were selected for resistance to G418 antibiotic. We isolated three clones (UV15/3T3, UV20/3T3 and UV25/3T3) expressing the mutant PKC gene and one clone (α -PKC/3T3) that expresses the normal mouse α -PKC gene. The UV25/3T3 cell line contains about ten times the level of high-affinity phorbol ester-binding sites as the control cells and shows a tenfold increase in PKC activity (Table 1). This cell line also contains elevated levels of PKC-specific messenger RNA transcripts (Table 2). The two other cell lines, UV15/3T3 and UV20/3T3, express medium levels of the mutant PKC gene and the α -PKC/3T3 cell line expresses high levels of α -PKC (Tables 1 and 2).

The UV25/3T3 transfectant (expressing the mutant PKC gene), like the fibrosarcoma cells, showed high membrane-associated PKC activity. By contrast, there was a normal PKC distribution of α -PKC/3T3 (expressing the mouse α -PKC cDNA) (Table 1). All transfectants, whether they express the mutant or the normal α -PKC gene, undergo morphological changes and display increased growth rate and saturation density. But comparison of their focus-forming activity revealed a difference in transforming potency (Table 2): the α -PKC gene fails to induce foci and the mutant PKC gene induces foci as early as 10 days after-transfection. The focus-forming activity of the mutant PKC gene was 4×10^5 units per pmol DNA, which is comparable with the efficiency of other known oncogenes¹⁷.

To compare further the transforming potential of the two PKC constructs, we tested the ability of the transfected cells to form colonies in soft agar. As shown in Fig. 3, when 2×10^4 cells are plated in 0.5% soft agar, the UV2240 fibrosarcoma and the UV15/3T3, UV20/3T3 and UV25/3T3 (expressing the mutant PKC-gene) form large colonies quite efficiently, whereas the control Balb c/3T3 transfected with pLTR vector or the parental Balb c/3T3 fail to grow and persist as single cells. The cells expressing the α -PKC gene form low and varying numbers of very small colonies. The colony-forming efficiency of UV25/3T3 was comparable to that of the tumorigenic UV2240 fibrosarcoma and to NIH/3T3 cells transformed by LTR/proto *abl* or LTR-driven human *c-sis*¹⁷.

TABLE 2 Focus-forming activity, anchorage-independent growth and tumorigenicity of cells transfected with mutant PKC cDNA

Cell line	Transfected construct*	Relative α -PKC mRNA level†	Focus-forming activity‡	Colony-forming efficiency (%)§	Tumorigenicity (tumours/injections)¶	Latency (d)	Tumours exceeding 3 cm diameter by 30 d#
Balb c/3T3	—	—	$<1 \times 10^0$	<0.1	0/10	—	0/20
Neo/3T3	pLTRNeo	—	$<1 \times 10^0$	<0.1	0/10	—	0/20
PKC/3T3	pLTRNeo/ α PKC	+++++	$<1 \times 10^0$	<5	0/20	—	0/20
UV2240	—	—	5×10^3	39	10/10	10–14	10/10
UV15/3T3	pLTRNeo/UV25	+	4×10^3	30	6/6	17–30	14/20
UV20/3T3	pLTRNeo/UV25	+	4×10^3	30	5/6	17–30	16/20
UV25/3T3	pLTRNeo/UV25	+++++	4×10^3	30	20/20	17–30	17/20
B-UV25/3T3	pLTRNeo/B-UV25	++++	$<1 \times 10^0$	<5	0/20	—	0/20

* For cotransfections, the PKC-expression plasmid was used at a 10:1 weight ratio over the construct bearing a selection marker.

† As estimated by qualitative visual analysis of Northern blots.

‡ Foci were scored from 7 to 16 days post-transfection.

§ All suspensions were plated at 10-fold serial dilutions in 0.3% soft agar medium as described in the legend to Fig. 3. Colonies comprising >100 cells were scored at 10 days.

¶ CD-1 nude mice were inoculated subcutaneously with 10^5 transfected cells. Tumorigenicity is expressed as the ratio of mice with tumours versus the number of mice inoculated. Tumours developed within 30 days.

|| Approximate time interval during which visually apparent tumours arose.

Data are expressed as the number of tumours ≥ 3.0 cm in size per number of injections.

Poly(A)⁺ mRNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde, 1% agarose gels, blotted onto nylon membrane and hybridized with the ³²P-labelled full-length UV25.1 cDNA²⁶.

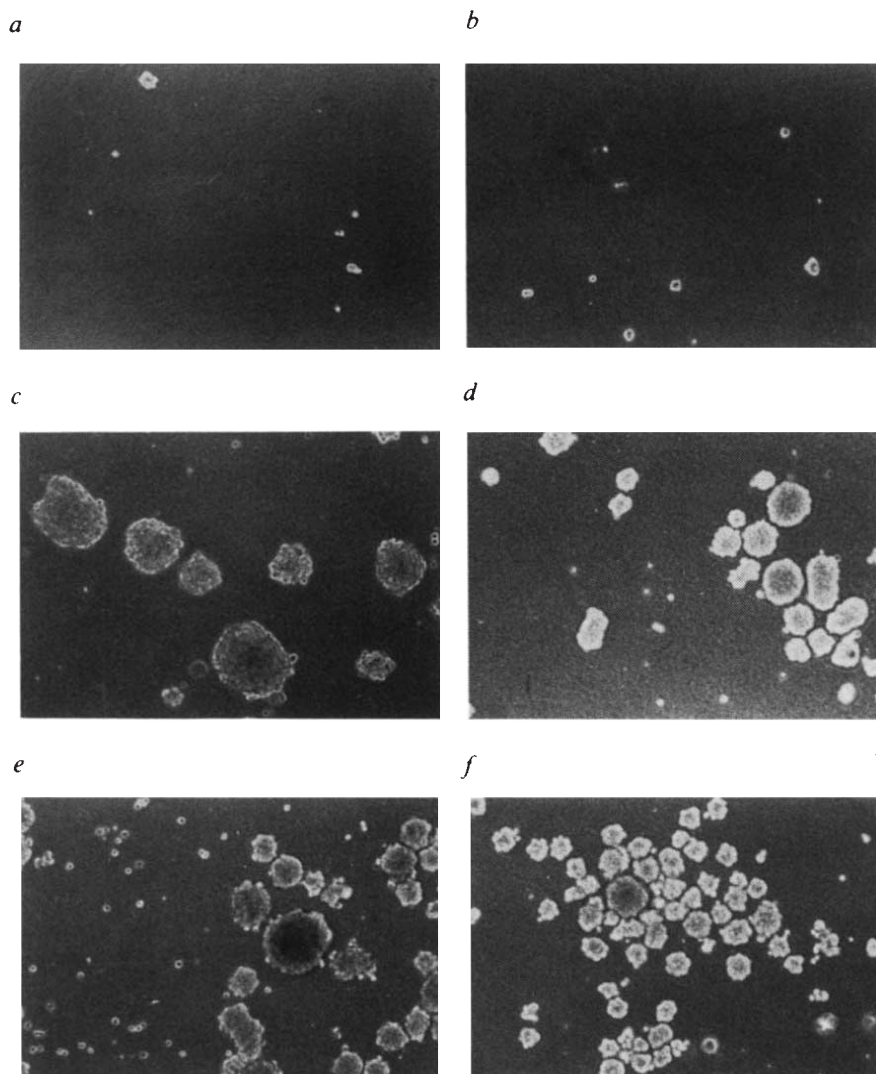


FIG. 2 Growth in soft agar. To assess anchorage-independent growth, 2×10^4 cells were suspended in 2 ml 0.3% Bacto-agar (Difco) in DMEM containing 20% fetal calf serum and overlaid above a layer of 5 ml 0.5% agar in the same medium on 60 mm Petri dishes. After 10 days, colonies were stained with the vital stain 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate (Sigma) for 48 h at 37 °C in an incubator with 5% CO₂, and photographed at a magnification of 13 $\times$. a, Balb c/3T3; b, α -PKC/3T3; c, UV2240; d, UV25/3T3; e, UV20/3T3; and f, UV15/3T3.

We have also measured the transforming properties of cells expressing the mutant PKC gene by their ability to produce tumours in nude mice (Table 2): 10^5 cells of the transfectants UV15/3T3, UV20/3T3 and UV25/3T3 could induce tumours in all injected nude mice within two weeks. Most of the tumours attained diameters ≥ 20 mm after 14 days and continued growing.

Histopathologically, all tumours were high-grade fibrosarcoma (data not shown). The UV25/3T3 tumour-derived cell lines (tumour cells re-established in culture) were resistant to G418 antibiotics and expressed high levels of mutant α -PKC. The tumorigenic potential of the UV25/3T3 cells is comparable with that of the *ras*-transformed fibroblasts¹⁸. By contrast, inoculation

of 10^5 Balb c/3T3, α -PKC/3T3 or 3T3 containing the pLTR expression vector alone did not form tumours even after two months (Table 2).

To investigate whether the transforming properties of the mutant PKC gene are due to the four point mutations in the coding region, we have back-mutated the transforming allele of the PKC gene. Oligonucleotide-directed mutagenesis was used to back-mutate the four point mutations in the mutated PKC cDNA and the region of each mutation was sequenced to verify the alteration. A back-mutated UV25 cDNA (B-UV25) clone was isolated and inserted into the pLTR expression vector. Balb c/3T3 cells were co-transfected with the back UV-25 cDNA and PSV-Neo plasmids. Transfected cells were selected for G418 antibiotic-resistance and we isolated one clone expressing the back-mutated PKC gene (B-UV25/3T3). The B-UV25/3T3 cell line contains about ten times the level of high-affinity phorbol-ester binding sites and its PKC activity is increased tenfold (Table 1). The revertant B-UV25/3T3 cell line, however, does not display features typical of malignant transformed cells (such as morphology, formation of dense foci and anchorage-independent growth) and fails to induce tumours *in vivo* when inoculated into nude mice (Table 2).

Our study establishes that the mutant PKC gene can be activated as a potent oncogene when its expression is driven by a strong promoter. Moreover, it is also evident that the mere expression, without overexpression, of the mutant α -PKC gene is sufficient to fully transform Balb c/3T3 fibroblasts. This mutated α -PKC gene differs from the α -, β - and γ -PKC-isoenzymes by four point mutations located in the regulatory and the catalytic domains. These results corroborate the findings of a number of distinct activating point mutations in the coding regions of the *ras*, *neu* and *fms* genes¹⁸⁻²⁰. It may be that a subset of proto-oncogenes, including the PKC gene, are activated after exposure to carcinogens or ultraviolet light, which act as point mutagens²¹. The idea of the PKC gene belonging to such a family is strengthened by the finding of a tumour-specific microscopic substitution in the α -PKC gene in a malignant melanoma primary cell line²². Oncogenic conversion of PKC may shed light not only on the process of carcinogenesis but also on the critical aspects of signal transduction and the mitogenic response. □

Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes

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THE lymphocyte function-associated molecule LFA-1 (CD11a/CD18) plays a key part in lymphocyte adhesion^{1,2}. Lymphocytes do not adhere spontaneously; activation of protein kinase C (PKC)³ by phorbol esters, however, gives rise to strong LFA-1-dependent adhesion⁴, indicating that activation of LFA-1 is required to induce cell adhesion. We have now investigated whether the functionally important CD2 and CD3 surface structures on T lymphocytes⁵⁻⁸ are involved in the activation of LFA-1. The stimulation of these molecules, which causes activation of PKC^{9,10}, strongly promoted LFA-1-dependent adhesion. Furthermore, we demonstrate by using cells from an LFA-1-deficient patient that this enhanced lymphocyte adhesion is caused by activation of the LFA-1 molecule and not by activation of its ligands. LFA-1 was persistently activated by triggering through CD2 but only transiently by triggering through CD3. We postulate that CD2 and CD3 can differentially regulate the affinity of LFA-1 for its ligands by modulating its molecular conformation through PKC-dependent mechanisms.

LFA-1, CR3 and p150, 95 comprise a group of heterodimeric adhesion receptors with a unique α -chain and a common β -chain. They mediate adhesion among leukocytes and of

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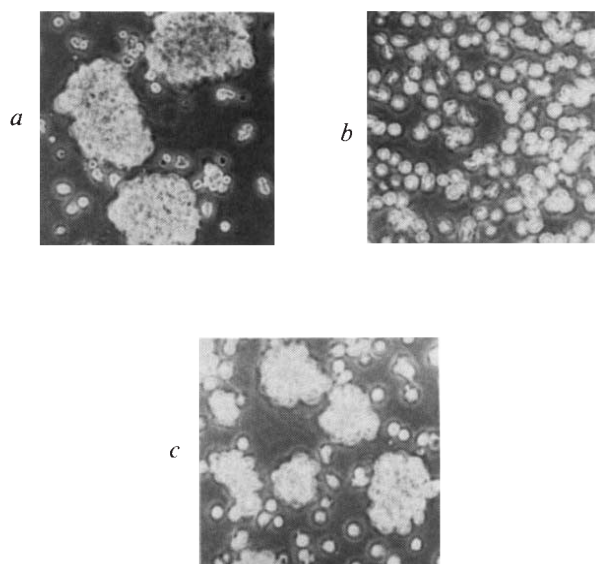


FIG. 1 Photomicrographs of homotypic cell adhesion and its inhibition by anti-LFA-1 antibodies. JS-136 cells were incubated with: a, NKI-L16 antibody ($1 \mu\text{g ml}^{-1}$); 80% adhesion; b, NKI-L16 ($1 \mu\text{g ml}^{-1}$) and anti-LFA-1 ($5 \mu\text{g ml}^{-1}$); <1% adhesion; or c, anti-CD3 ($10 \mu\text{g ml}^{-1}$; 60% adhesion), and scored in the qualitative assay (Table 1) for 40 min.

TABLE 1 Homotypic cell adhesion of JS-136 T cells and NL-3 NK cells

	JS-136			NL-3		
	Medium	+Anti-LFA-1	+Anti-LFA-3	Medium	+Anti-LFA-1	+Anti-LFA-3
Medium	10	<1	10	10	<1	10
TPA	60	<1	60	80	<1	80
NKI-L16	80	<1	80	70	<1	70
Anti-CD3	60	<1	60	10	<1	10
Anti-CD2	70	<1	60	60	<1	60
Anti-CD4	10	<1	10	10	<1	10
Anti-CD71	10	<1	10	10	<1	10

Induction of homotypic cell adhesion (%) of JS-136 T cells and NL-3 NK cells after stimulation with TPA, mAbs NKI-L16, anti-CD3 and anti-CD2, and with isotype-matched control antibodies directed against CD4 and the transferrin receptor (CD71). Stimuli were added in the following concentrations: TPA (10 ng ml⁻¹), NKI-L16¹⁵ (1 µg ml⁻¹), anti-CD3 antibodies²⁵ (SPV-T3b; 10 µg ml⁻¹), a combination of anti-CD2 antibodies¹⁷ (CLB-T11.1/1 clone 6G4 and CLB-T11.2/1 clone 4B2; 10 µg ml⁻¹), anti-CD4 (CLB-T4/1; ref. 26) and anti-CD71 (661G10; 10 µg ml⁻¹; ref. 27). Cell adhesion was blocked after addition of anti-LFA-1 antibodies¹⁹ (CLB LFA-1/1; 5 µg ml⁻¹) or anti-ICAM antibodies (RR 1/1; data not shown). Addition of anti-LFA-3 antibodies (TS 2/9; 5 µg ml⁻¹) did not inhibit cell adhesion. One representative experiment out of four performed is shown (s.d. <10%). All experiments were carried out in triplicate (s.d. <10%). JS-136 (CD2⁺ CD3⁺ CD4⁺ LFA-1⁺ LFA-3⁺ ICAM-1⁺) is a cytolytic T-cell clone, directed against HLA-DR²⁸. The NK clone (NL-3) expressed the following surface molecules: CD3⁺ CD2⁺ LFA-1⁺ LFA-3⁺ ICAM-1⁺. Cells were cultured as described previously²⁸ and tested 4 days after re-stimulation when spontaneous cell aggregation was ≤10%. Homotypic cell adhesion was measured as described previously¹⁵. Before the test, wells were washed twice in Iscove's medium containing 10% FCS, resuspended, and 2 × 10⁵ cells seeded in 96-well microtitre plates in a volume of 50 µl. Monoclonal antibodies were added in a volume of 50 µl and the cells were incubated for 40 min at 37 °C, after which aggregate formation was determined through light microscopy by at least two investigators. Scores ranged from <1–100, in a scale of 10, where <1 indicated that essentially no cells were aggregated in clusters and 10, 20, 40, 60, 80 and 100 correspond with cell aggregations of <10%, <30%, <50%, <80%, >80% in compact clusters, and >90% large compact clusters, respectively. The percentage aggregation was determined by counting the number of free cells, using the equation: percentage of aggregation = 100 × [1 – (no. free cells/no. input cells)]; no. of input cells = no. cells per ml in control tube containing only cells and medium; no. free cells = no. nonaggregated cells per ml from experimental tube.

leukocytes with other cell types by binding to their respective ligands. Defined ligands of LFA-1 are the intercellular adhesion molecule-1 (ICAM-1, CD54)¹¹ and -2 (ICAM-2)¹². Leukocytes do not spontaneously aggregate; activation of PKC by phorbol esters, such as TPA (12-*O*-tetradecanoylphorbol-13-acetate), however, can induce LFA-1- and CR3-dependent adhesion^{4,13–14}. An anti-LFA-1 monoclonal antibody (NKI-L16) or its Fab fragments, reactive with a unique epitope on the α-chain, is also capable of inducing LFA-1-dependent adhesion with kinetics strikingly similar to those seen with TPA¹⁵. These observations indicate that LFA-1-dependent adhesion requires a change of the conformation of LFA-1.

This prompted us to investigate whether the T-cell receptor-CD3 complex, responsible for recognition of antigen⁵, or CD2, which is believed to contribute to cell adhesion by binding to the LFA-3 molecule^{6–8}, can modulate LFA-1-mediated adhesion. The binding of ligands by these glycoproteins generates second messengers mediating the activation of PKC^{9–10}. Assuming that PKC can activate LFA-1, triggering of the CD2 or T-cell receptor-CD3 pathways should therefore result in T-cell adhesion¹⁶. Anti-CD3 monoclonal antibodies (mAbs) or a combination of two anti-CD2 mAbs required for T-cell proliferation¹⁷, added in soluble form or bound to plastic, indeed strongly enhanced homotypic aggregation of cells of an alloreactive human T-cell clone, JS-136 (Fig. 1; Table 1). Furthermore, anti-CD2 but not anti-CD3 mAbs promoted homotypic adhesion of CD3⁺ NK clone (NL-3) cells, whereas TPA or NKI-L16 induced adhesion of both CD3⁺ and CD3⁺ cells, without an apparent increase in expression of LFA-1 or ICAM-1. Isotype-matched control antibodies directed against other surface molecules expressed by these clones were ineffective at inducing adhesion, indicating that the adhesion induced by anti-CD3 or anti-CD2 mAbs is not caused by agglutination. The induced adhesion is mediated by LFA-1 because it can be inhibited by anti-LFA-1 antibodies, but not by anti-LFA-3 antibodies. This notion is supported by the characteristic Mg²⁺ dependence and temperature dependence of LFA-1-mediated adhesion (data not shown).

The kinetics of aggregation were examined next (Fig. 2). Maximal aggregation of JS-136 cells was found within 20 min, regardless of whether the cells were stimulated with TPA, NKI-L16 or mAbs anti-CD3 or anti-CD2. To measure the time course of LFA-1 activation, aggregates were disrupted at various time-points after initiation of adhesion, and the ability of the suspended cells to re-form aggregates was determined. Cells stimulated by TPA, NKI-L16 and mAb anti-CD2 were able to

re-aggregate without extra addition of stimuli, and remained maximally aggregated for >12 h. By contrast, the anti-CD3-induced aggregates were unable to re-form aggregates when the cells were resuspended later than 30 min after initial induction (Fig. 2), indicating that anti-CD3 mAb transiently activates

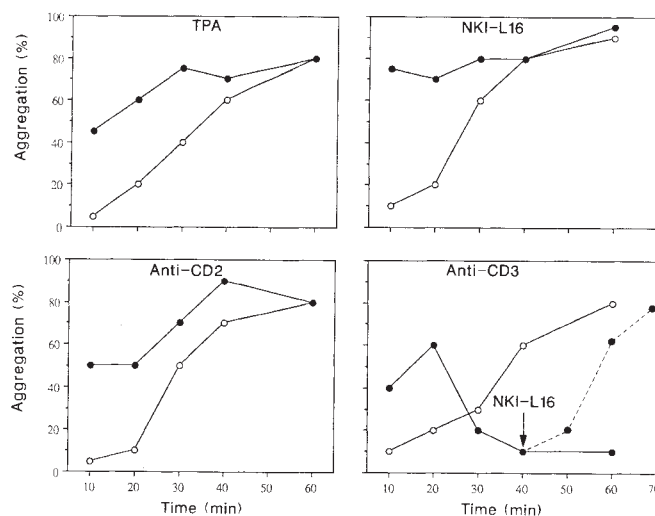


FIG. 2 Kinetics of homotypic cell adhesion of T-cell clone JS-136 (%) induced by TPA, or mAbs NKI-L16, anti-CD2 or anti-CD3. Cell adhesion was followed in time (—○—) and the capacity of cells to re-aggregate after disruption of cell clusters (—●—) is shown. Cells were stimulated for various periods of time. Subsequently, aggregates were disrupted, and the % aggregation was determined after an additional incubation period of 40 min without extra addition of stimuli (—●—). The inability of anti-CD3-treated cells to re-aggregate could be completely restored by the addition (arrow) of NKI-L16 antibody (---●---).

METHODS. Homotypic cell adhesion of cytotoxic T-lymphocyte clone JS-136 cells was performed as described in Table 1. The percentage of aggregated cells was determined in the course of time, and the concentrations of mAbs used (—○—) are given in Tables 1 and 2. Activation of LFA-1 was measured at various time intervals (—●—). Cell clusters were disrupted by vigorous pipetting to form single-cell suspensions. Subsequently the capacity of cells to re-aggregate was measured without the addition of extra stimuli (experiments were carried out in triplicate (s.d. <10%; one representative experiment out of four performed is shown, s.d. ≤10%). Nonspecific aggregation (spontaneous aggregation and aggregation induced by control antibodies of IgG1 and IgG2a isotype) was always <10%.

TABLE 2 Heterotypic cell adhesion of LFA-1⁺ with LFA-1⁺ T-cell clones

T-cell clones		Stimulus	Heterotypic aggregation (%)
LFA-1 ⁺ LAD 6 pretreatment	LFA-1 ⁺ JS-136 pretreatment		
—	—	—	10
—	—	NKI-L16	70
—	—	TPA	40
—	—	Anti-CD3	40
—	—	Anti-CD2	50
NKI-L16	—	—	<1
TPA	—	—	<1
Anti-CD3	—	—	<1
Anti-CD2	—	—	<1
—	NKI-L16	—	60
—	TPA	—	40
—	Anti-CD3	—	40
—	Anti-CD2	—	40

Heterotypic cell adhesion of cloned LFA-1⁺ T cells (LAD 6) with LFA-1⁺ T cells (JS-136). Heterotypic cell adhesion was determined 90 min after addition of medium, NKI-L16 ($1 \mu\text{g ml}^{-1}$), TPA (10 ng ml^{-1}), anti-CD3 (SPV-T3b; $10 \mu\text{g ml}^{-1}$) and anti-CD2 (T11₁ + T11₂, $10 \mu\text{g ml}^{-1}$). Pre-incubation of LAD 6 with NKI-L16, TPA, mAbs Anti-CD3 or anti-CD2 did not result in the formation of cell clusters, whereas pre-incubation of JS-136 cells caused formation of heterotypic aggregates indicating that adhesion is induced through LFA-1. TPA, or mAbs NKI-L16, anti-CD3 or anti-CD2 did not induce homotypic aggregation of LAD 6 cells (data not shown). A representative experiment out of three is shown (s.d. < 10%); experiments were carried out in triplicate (s.d. < 10%). The LFA-1⁺ T cell clone LAD 6 (CD2⁺ CD3⁺ CD4⁺ CD8⁺ LFA-3⁺ ICAM⁺) was raised by limiting dilution of peripheral blood lymphocytes (PBL) from a LAD patient. Clones were grown weekly on stimulation with irradiated allogeneic PBL and cells of the B-cell line JY, supplemented with phytohaemagglutinin (PHA; $0.2 \mu\text{g ml}^{-1}$) and interleukin 2 (IL-2; 100 U ml^{-1}). Heterotypic cell adhesion was determined by means of double fluorescence. LAD 6 cells ($1 \times 10^6 \text{ ml}^{-1}$) were stained with red hydroethidine (HE; Polyscience, in *N,N*-dimethylacetamide) at a concentration of 3 mg ml^{-1} in Iscove's medium. JS-136 cells ($1 \times 10^6 \text{ ml}^{-1}$) were stained with the green dye (sulphofluorescein diacetate (SFDA) in dimethyl sulphoxide; Molecular Probe) at a concentration of $5 \mu\text{g ml}^{-1}$. After 1 h incubation at 37°C , cells were washed twice with Iscove's medium containing 10% FCS and seeded in flat-bottomed plates in a concentration of 1×10^5 HE-labelled cells together with 1×10^5 SFDA-labelled cells per well. Aggregation was performed at 37°C , and after 90 min the cells were fixed in 0.5% paraformaldehyde followed by fluorescence microscope analysis to determine the percentage of heterotypic cell aggregates. The aggregates were checked to be composed of equal ratio of the two different cell types. In those cases where LAD 6 cells or JS-136 cells were pretreated with NKI-L16, TPA, mAbs anti-CD2 or anti-CD3 for 20 min at 37°C , the cells were extensively washed to remove nonbound antibodies.

LFA-1, and that CD2 and CD3 could activate LFA-1 through different signalling pathways. The transient CD3-mediated response was not due to decreased expression of LFA-1 or its ligand(s), because these anti-CD3-treated cells could still be induced to aggregate by NKI-L16 antibodies (Fig. 2). When, instead of activated T cells, resting peripheral blood lymphocytes were used, a maximal aggregation was only observed after 6 h incubation. This seems to be directly correlated with the expression of the NKI-L16 epitope on the LFA-1 molecule (Y.v.K., unpublished results).

To prove that induction of adhesion is a consequence of activation of the LFA-1 molecule, rather than activation of its ligand(s) or other adhesion molecules (CD2-LFA-3), we performed heterotypic cell-binding studies with LFA-1-positive (LFA-1⁺) and LFA-1-deficient (LFA-1[−]) T cells of a patient suffering from leukocyte adhesion deficiency syndrome^{1,2,18}. Leukocytes from these patients lack cell-surface expression of all CD11 and CD18 molecules, generally owing to a genetic defect in the common β -chain gene. A T-cell clone derived from this 'severe-type' leukocyte adhesion deficient patient completely lacked β -chain messenger RNA expression and LFA-1, CR3 and p150,95 surface expression. But T cells expressed normal levels of ICAM-1, CD2, CD3 and LFA-3 (data not shown). LFA-1[−] cells could not be induced to adhere among themselves, but were able to bind to JS-136 (CD3⁺ LFA-1⁺) cells after addition of TPA, or mAbs NKI-L16, anti-CD3 or anti-CD2 (Table 2). When the LFA-1[−] cells were incubated with the different stimuli, before mixing with the LFA-1⁺ cells, neither homotypic (among LFA-1[−] or LFA-1⁺ cells) nor heterotypic adhesion was observed. By contrast, pre-incubation of the LFA-

1⁺ cells with the same stimuli resulted in the formation of large clusters of cells containing both LFA-1⁺ and LFA-1[−] cells in a ratio of 1:1 (Table 2). These results show that T-cell adhesion is induced by activation of LFA-1 and not by activation of its ligands. Induction of heterotypic T-cell adhesion by these stimuli exclusively activates the LFA-1-mediated adhesion pathway and not the CD2-LFA-3 adhesion pathway, despite the fact that the LFA-1[−] T cells express normal levels of LFA-3 and CD2.

Evidence is thus presented for the existence of an activated form of LFA-1. Binding of NKI-L16 to LFA-1, or stimulation of PKC by TPA, or mAbs anti-CD2 or anti-CD3, probably results in a conformational modification of the LFA-1 molecule, leading to high-affinity ligand binding. Although induction of adhesion by NKI-L16 is thought to be exclusively an extracellular event, the molecular alteration induced by the other stimuli would be catalysed by PKC. This notion is supported by the observation that PKC inhibitors completely block TPA- or anti-CD3-induced aggregation, but have no effect on induction of adhesion by NKI-L16 (Y.v.K. *et al.*, unpublished results). Alternatively, LFA-1 itself could be involved in signal transduction¹⁹.

We propose that numerous LFA-1-dependent T-lymphocyte interactions occurring in an immune response are controlled by the T-cell preceptor-CD3 complex and by CD2. A case in point is the interaction between cytotoxic T lymphocytes and their target cells. It has been reported that LFA-1-dependent binding of the effector T cell to the target cell precedes recognition by the T-cell receptor-CD3 complex²⁰. The results presented here indicate that this cell-cell interaction, although of low affinity, facilitates antigen recognition by the T-cell receptor-CD3 complex. On antigen recognition, PKC, activated through CD3, could activate LFA-1 by direct phosphorylation of the β -chain of LFA-1^{21,22}, or alternatively, by phosphorylation of a cytoskeletal protein such as talin²³, which then might bind and activate LFA-1. This strengthens the interaction between cytotoxic lymphocytes and target cells for efficient delivery of cytotoxic mediators causing target-cell lysis. Target-cell detachment could also be controlled by PKC. Phosphorylation and subsequent modulation of CD3²⁴ possibly results in downregulation of PKC, and could thus explain the inactivation of LFA-1 observed 20 min after triggering with anti-CD3 antibody. □

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Sound-induced motility of isolated cochlear outer hair cells is frequency-specific

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THE inner ear is capable of highly selective frequency discrimination. This is achieved not only by the travelling wave of the basilar membrane in the cochlear partition, but also by the active participation of nonlinear and vulnerable elements that enhance frequency selectivity. It has been shown that isolated mammalian outer hair cells respond with a change in length when subjected to sound stimulation at a fixed frequency¹. Here we investigate the motile behaviour of isolated cells when the stimulus frequency is varied between 200 and 10,000 Hz. By varying the frequency and the intensity of the tone, it is possible to obtain 'tuning curves' for the motile response. We demonstrate that the cell body of solitary hair cells, free from contact with the basilar membrane, shows a sharply tuned motile behaviour. We suggest that frequency selectivity in the organ of Corti is amplified by the tuned motility of the cell body of outer hair cells.

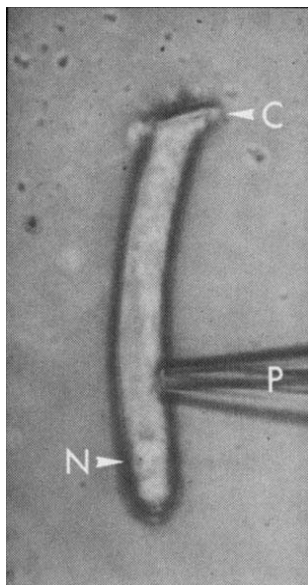


FIG. 1 Outer hair cells were stimulated by sound delivered to the cell membrane by a fluid-filled glass pipette. Cuticular plate (C), nucleus (N), pipette (P).

METHODS. Pigmented guinea pigs were decapitated and their temporal bones were placed in culture medium (Leibovitz' L-15; GIBCO). The appropriate region of the organ of Corti was gently removed from the basilar membrane. After 3 min incubation in collagenase (1 mg ml⁻¹ Sigma), the cells were separated by suction into a pipette, and placed in a 250-μl chamber containing L-15 medium (concentrations in mM: Na⁺, 135; K⁺, 5.8; Ca²⁺, 1.26; Cl⁻, 147; solution was 300 milliosmolar, pH was 7.4). Cells were viewed in phase contrast in a Reichert inverted microscope equipped for video microscopy (magnification, 1,200×). The cell was attached to the flame-polished 3–4 μm opening by applying negative pressure (<40 Pa) during stimulation. The purpose of applying this slight negative pressure was to hold the cell in a stable position, and not to create a gigaohm seal. The sound stimulus was generated by a Somedic vibrator, and was delivered by a Brüel and Kjaer minishaker (type 4810) driving a hydraulic system ending with a fluid-filled glass pipette. The stimulus consisted of a tone burst for 800 ms and a rise/fall time of 80 ms. The signal level could be controlled in 1.5 dB steps. Only healthy-looking cells were used in the study. Experiments were performed within 3 h of decapitation and at 23 °C.

A growing number of reports on inner ear physiology suggest that the active properties of the inner ear are involved in determining its frequency selectivity. The existence of a cochlear amplifier that increases the resolution of auditory information has been proposed^{2,3}. Length changes of isolated outer hair cells have been observed in response to chemical^{4,5} and electrical^{6,7} stimuli and it has been found that their acoustic stimulation at a fixed frequency elicits motile responses¹. In this study we varied the frequency of the stimulus to determine the frequency with the lowest threshold for the motile response (in Figs 1 and

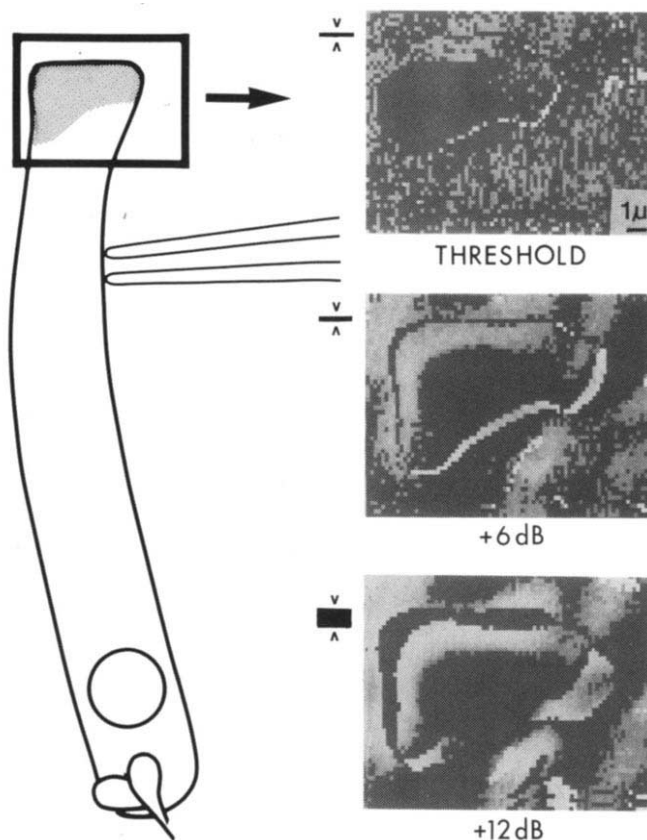


FIG. 2 Difference in the cell length determined by a computerized subtraction technique. The cell image obtained during stimulation was subtracted from the resting-state image. Owing to the light scattering of the cuticular plate, the difference between the two states is manifested as a dark band at the top of the cuticular plate and a light band at the bottom. Subtraction images are shown for three different stimulus intensities: at threshold, at 6 dB above threshold, and at 12 dB above threshold. The magnitude of the difference between the two states is indicated as a bar to the left of each subtraction image.

METHODS. Calibration: laser interferometry²³ was used to measure the vibration amplitude at the tip of a 10-μm pipette. This was measured in two ways. First, the laser beam was aimed at the opening of the pipette measuring the vibrations of the oscillating fluid. Second, a 100-μm glass bead was placed on the pipette tip, where it was held in place by contact with the fluid in the pipette. The amplitude of the oscillations was in both cases between 10⁻¹⁰–10⁻⁹ m. These values are higher than those applied to the cell because a larger pipette (10 μm) had to be used. The force produced by the sound field projecting from the capillary tip was measured using a Brüel and Kjaer 8001 force gauge. The force was less than 5 × 10⁻¹³ N. The distribution of the fluid vibration at the pipette opening was explored by investigating the movements of 1.23 μm latex beads. The distribution was symmetrical and cone shaped. The amplitude of the driving shaft of the vibrator was measured by a photodiode and was found to be flat (±3 dB) between 400–10,000 Hz, and insensitive to variations in pipette-opening diameter. Calibration was also achieved by determining the threshold for stereocilia motion in stroboscopic light¹². Results were consistent with photodiode calibration, although limited to 4,000 Hz on the high-frequency side because of flash-duration limitations. Higher motility thresholds seen for high-frequency cells could be due to a drop in output at the pipette tip.

2). We find that this best frequency is related to the cochlear position from which we obtained the cell.

Acoustic stimulation elicited a length-change in 76% of the cells studied ($n = 36$). The motile response of each cell varied with frequency and demonstrated optimal sensitivity in a narrow frequency region (Fig. 3). Above and below this region, the motile response was gradually lost, and as a consequence the stimulus intensity had to be increased to elicit a response. By varying the frequency and the intensity it was possible to determine a tuning curve for each individual cell. The sharpness of tuning can be expressed by the $Q_{10\text{dB}}$ value, which is obtained by dividing the best frequency by the bandwidth, measured 10 dB above the most sensitive region. The $Q_{10\text{dB}}$ values for isolated outer hair cells are similar to those obtained from single fibre and compound action potential recordings from the eighth nerve⁸. The best frequency of each cell was related to its original position in the cochlea (Fig. 4). There is a correlation factor of 0.91 between hair cell length and best frequency. The dissection of the basal part of the cochlea (4–10 mm from the round window), excluding the hook region, yielded short cells (20–50 μm) showing a motile response to frequencies between 3,000–10,000 Hz, whereas dissection of the apical regions (13–19 mm from the round window) gave long cells (75–95 μm) responding to frequencies below 1,000 Hz. This corresponds to the known frequency distribution in the cochlea^{9–11}. The majority of the cells (31) shortened in response to stimulation, and all cells with a best frequency >1,000 Hz exhibited a shortening response. At very high frequencies (>7,000 Hz), the shortening consisted of a tilting movement of the cuticular plate and a constriction beneath this region. Long cells of the apical turn could either shorten or elongate. Five cells elongated, and showed tuning of equal sharpness. The direction of motion was unaltered when the frequency was varied. The pipette was generally aimed at

the mid-portion of the basolateral cell membrane, but length changes of equal direction and magnitude could be elicited anywhere along the lateral side of the outer hair cell.

The magnitude of the movement followed the envelope of the tone, and was graded with stimulus intensity (Fig. 2). To estimate the relative intensity of the stimulus, the stimulating pipette was aimed towards the stereocilia at a 5 μm distance in the isolated coil preparation^{12,13}, and intensity thresholds for stereocilia movement were studied by the use of stroboscopic light. The threshold intensity needed to elicit a length change of the cell was generally lower (5–10 dB) than the intensities needed for the detection (0.2 μm) of stereocilia deflection. The magnitude of hair cell motion was 0.5–2.0 μm at 9 dB above threshold and depended on cell length, smaller movements being seen in the short high-frequency cells. As previously shown¹, the addition of 3 μM poly-L-lysine (molecular weight 8,000–15,000) to the bath inhibited the motile response. In some cases the pipette was seen to instantly vibrate at a particular frequency, but this vibration did not relate to the motility frequency of the cell, and such movement could be separated from the biological motility by its insensitivity to polylysine. Vital outer hair cells appear stiff, rod-like and glistening. Stiffness was easily tested by a gentle touch of the pipette. Among the healthy-looking cells, we found some that had lost their stiffness without losing shape. These soft cells were unresponsive.

The sharpness of frequency selectivity in the hearing organ is expressed in the action potential of the auditory nerve¹⁴, in the a.c. and d.c. receptor potentials of hair cells^{10,11}, and in the mechanical vibration of the basilar membrane¹⁵ and the reticular lamina¹⁶. The tuned property is distributed along the cochlear partition and is commonly explained by the travelling wave¹⁷. We demonstrate that the sensory cells of the inner ear *per se* are sharply frequency-selective in the absence of the basilar membrane. The best frequency of each cell was clearly correlated to the length of the cell and to its original cochlear position. This indicates that the sharply tuned portion of the tuning curve, often referred to as the tip, is independent from the basilar membrane travelling wave. The sharp tuning is, rather, a result of the inherent tuning of individual hair cells and the tonotopic distribution of these cells within the organ of Corti. This is supported by recent findings that the amplitude of motion of the reticular lamina exceeds that of the basilar membrane¹⁶, and that manipulation of outer hair cell motility affects the vibration pattern of the cochlear partition¹⁸. The shift of basilar membrane position in response to sound stimulation¹⁹ may also reflect outer hair cell motility. The sharp sensitivity in frequency

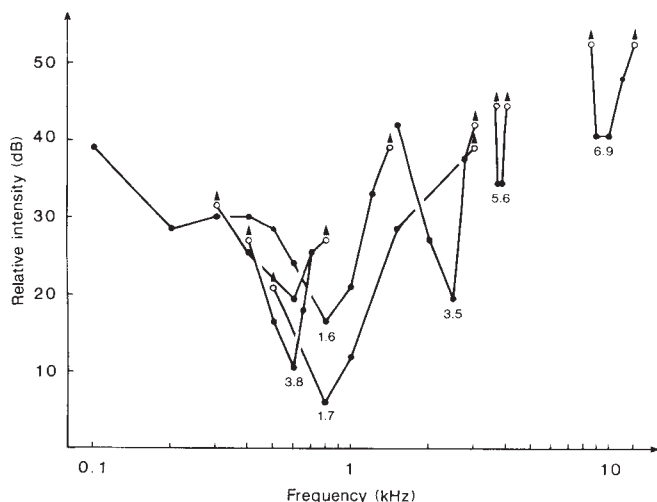


FIG. 3 Motility tuning curves obtained from seven outer hair cells dissected from different cochlear locations; the same glass pipette was used so that relative intensities were comparable. Open circles indicate absence of response at a tested frequency and intensity. Arrows indicate that the actual threshold must be at higher intensity. The sharpness of each tuning curve is represented by the $Q_{10\text{dB}}$ value under each curve. All cells except the one with a $Q_{10\text{dB}}$ of 1.7 showed a shortening response. The dB scale is relative, and the zero level was set at a driver amplitude of 15 mV, corresponding to a vibration amplitude lower than 10^{-9} M.

METHODS. Each cell was screened for its motile response by varying the frequency: in 100 Hz steps between 200–1,000 Hz, and 1,000 Hz steps between 1,000–10,000 Hz, or by sweeping across the frequency range. When a motile response was observed, stimulus intensity was decreased to obtain a visual detection threshold, defined as the smallest stimulus that elicited a detectable motile event. The visual detection threshold corresponds to a length change of ~ 0.1 μm . This was confirmed by subtraction images at threshold intensity. The screening of each cell ended by returning to the cell's most sensitive frequency region to confirm its responsiveness.

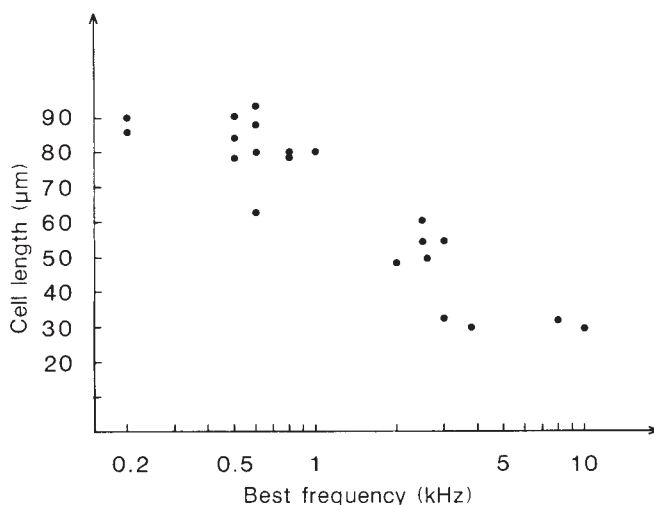


FIG. 4 Correlation between the length of the outer hair cells and their best frequency. Long cells, obtained from the apical parts of the cochlea, responded best to low frequency stimulation, whereas shorter cells dissected from more basal regions responded best to higher frequencies.

resolution is lost when outer hair cells are damaged^{20,21}.

The sound energy acting on the basolateral membranes of the outer hair cell can be transduced into a length change. The motile function of the outer hair cells could thereby provide a means for frequency tuning in the cochlea, overriding the low-pass filtering properties of the hair cell membranes²².

Our results can be summarized: (1) Outer hair cells can sense the mechanical vibration of the cell wall and respond by a change in cell length; (2) at a fixed frequency, the degree of change is graded with stimulus intensity; (3) the stimulus level required to induce a threshold change varies with frequency. The intensity is minimal at the best frequency, and increases both above and below this frequency. The single isolated hair

cell therefore displays a sharply tuned mechanical response; (4) the frequency to which an outer hair cell is tuned is correlated with the cell length. Long cells respond best to low frequencies and short cells respond best to high frequencies; (5) the best frequency of an outer hair cell depends on the region of the cochlea from which it was obtained. Cells from the apical turn respond best to low frequencies, whereas cells from the basal turn are most sensitive to high frequencies. These observations show that each outer hair cell is a tuned detector of mechanical vibrations. Each hair cell is specifically tuned to detect signals over a narrow range of frequencies, and this selectivity arises from the mechanical tuning of the cell body of the outer hair cell. □

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Specific binding of HIV-1 recombinant Rev protein to the Rev-responsive element *in vitro*

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THE human immunodeficiency virus type 1 (HIV-1) genome encodes the regulatory protein Rev, of relative molecular mass 13,000, which is synthesized from fully processed viral transcripts before synthesis of HIV-1 structural proteins^{1–3}. Rev has been postulated to exert control within the nucleus at the level of messenger RNA processing^{4–7}. The availability of Rev in the nucleus serves to increase the proportion of unspliced and singly spliced mRNA species relative to fully spliced mRNA molecules, resulting in an increased synthesis of viral structural proteins. A highly conserved *cis*-acting sequence termed the Rev-responsive element (RRE) has been identified in the envelope gene (*env*) of the viral transcript that seems to control mRNA processing in a Rev-dependent manner^{8–11}. Genetic studies have identified *rev* gene mutants with dominant phenotypes, supporting the hypothesis that Rev interacts directly with the RRE¹². Here we demonstrate that Rev protein, purified from *Escherichia coli*, binds in a sequence-specific manner to the RRE element *in vitro*.

Radiolabelled RNA transcripts containing the RRE were synthesized *in vitro* using T7 or T3 RNA polymerase (Fig. 1) for use in both gel-shift and nitrocellulose-filter-binding assays. For gel-shift assays, a 367-nucleotide 'sense' RNA fragment containing the RRE was used. A similarly sized 'antisense' RNA fragment expected to possess significant secondary structure was used as a nonspecific control fragment. The complementary DNA clone for *rev* (ref. 13) was expressed in *E. coli* as a nonfusion protein. MS2 viral RNA was used as an unlabelled nonspecific competitor RNA species. The Rev protein was purified to >95% homogeneity by ion-exchange and gel-filtration chromatography.

The addition of Rev to the sense RNA-containing sample

decreased the gel mobility of the labelled RNA species (Fig. 2). The observed mobility shift was dependent on Rev concentration and reflected a specific Rev-RRE interaction, because a large excess of unlabelled transfer RNA (5 µg) was included in each sample to eliminate nonspecific RNA binding. No Rev binding to the antisense RNA was detected under identical conditions. In addition to the main protein-nucleic acid complex (Fig. 2, large arrow), several other specific Rev-RRE complexes of intermediate mobility were observed (small arrows). Most of the bound RNA was contained in the lower-mobility complex at all of the observed Rev concentrations and could indicate multiple Rev molecules binding to the RRE, with intermediate bands representing less-stable incomplete complexes. Addition of either 25 µg unlabelled tRNA or 2×10^{-7} M unlabelled RNA containing the RRE to each sample caused no change in the

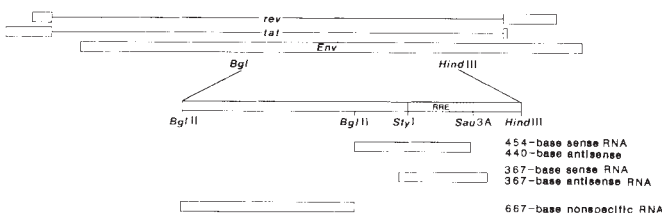


FIG. 1 Map of the HIV *rev*, *tat*, and *env* genes showing RNAs synthesized *in vitro*. The DNA fragments indicated were subcloned from a genomic HIV clone¹⁵ into plasmid pBluescript SK minus (Stratagene). The RNAs produced *in vitro* are composed of the indicated fragment of the HIV *env* gene as well as some sequence derived from pBluescript and are named according to the length of the RNA in nucleotides. The nucleotide sequence of the region between the *StyI* and *Sau3A* sites was determined and corresponds to that of HXB3. The 367-base sense and antisense RNAs contain the HIV sequence corresponding to nucleotides 7,333–7,612; the 454-base sense and 440-base antisense RNAs contain nucleotides 7,199 to 7,569; and the 677-base nonspecific RNA contains nucleotides 6,619–7,202. The restriction sites shown on the HIV envelope gene fragment are *BglII* (6,618); *BglII* (7,198); *StyI* (7,356); *Sau3A* (7,566) and *HindIII* (7,718). Sequence coordinates are according to Ratner *et al.*¹⁶.

METHODS. RNAs were synthesized *in vitro* using T7 or T3 RNA polymerases (Stratagene) according to the supplier's protocol, except that the UTP concentration was decreased to 130 µM and [α -³²P] UTP (NEN) was included at 45 Ci mmol⁻¹. Reactions were terminated by DNase digestion followed by phenol-chloroform extraction. Unincorporated nucleotides were removed by chromatography through Sephadex G-50.

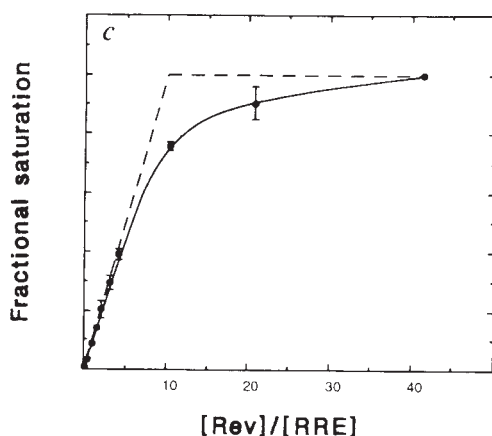
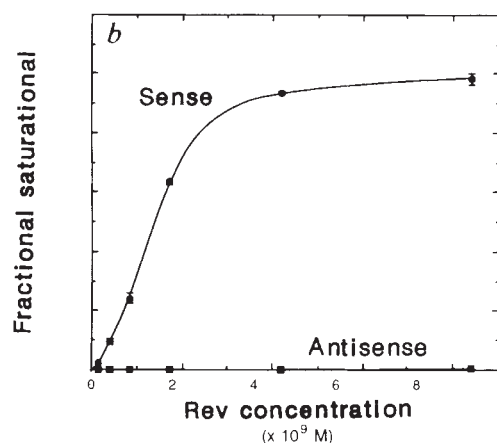
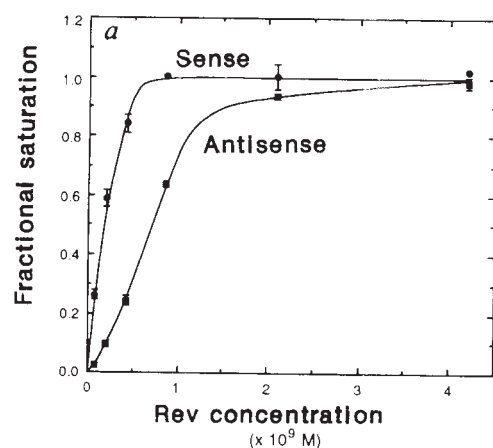
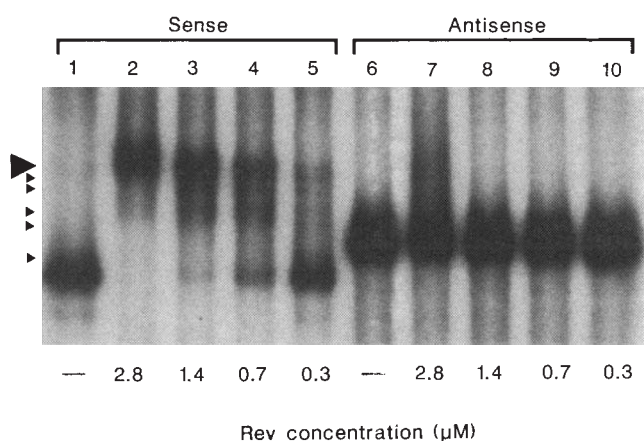


FIG. 2 Specific binding of Rev to an RNA fragment containing the RRE by gel-shift assay. Rev concentrations were as follows: lanes 1 and 6, 0 M; lanes 2 and 7, 2.75×10^{-6} M; lanes 3 and 8, 1.37×10^{-6} M; lanes 4 and 9, 6.87×10^{-7} M; lanes 5 and 10, 3.43×10^{-7} M. Arrows indicate Rev-dependent altered mobility of RNA.

METHODS. HIV cDNA encoding the *rev* gene¹³ was cloned into plasmid vector pKK233-2 (Pharmacia) using the *Nco*I and *Hind*III restriction sites of the vector and synthetic oligonucleotide linkers. The resulting plasmid, pKKREV, was maintained in *E. coli* strain XL1-Blue (Stratagene) and recombinant protein expressed by induction of log-phase cells with 1 mM isopropyl- β -D-thiogalactoside (IPTG). Disrupted cells were purified by solubilization in urea and fractionation by carboxymethyl-Sepharose chromatography and S-200 (Pharmacia) gel filtration in urea-containing buffers. Purified Rev was renatured by dialysis in PBS before use. The purified Rev was judged more than 95% pure by SDS-PAGE and Coomassie-blue staining. Purified Rev was stored at -20°C in PBS buffer. Gel-shift assay: Samples containing RNA and protein were electrophoresed on a standard TBE 5% polyacrylamide gel using a variation of the method of Fried and Crothers¹⁷. All samples contained 5 μg unlabelled tRNA (Boehringer), 10 U RNasin (RNase inhibitor; Promega), and either the ^{32}P -internally labelled 367-base sense RRE-containing RNA (lanes 1-5), or the similarly sized ^{32}P -internally labelled antisense RNA (lanes 6-10) in PBS buffer. The final volume of each sample loaded onto the polyacrylamide gel was 10.5 μl . Samples were electrophoresed for ~ 2 h at 15 mA. Gels were dried before autoradiography.

protein-RNA complex banding pattern (data not shown). This indicates that the dissociation constant under these reaction conditions for gel retardation is $> 2 \times 10^{-7}$ M. On the basis of these results it is concluded that either a single Rev molecule interacts with the RRE-containing fragment and elicits multiple RNA conformations that can be detected by gel shift, or multiple Rev molecules are capable of interacting specifically with the RRE.

Traditionally, protein-dependent retention of nucleic acids on nitrocellulose filters has been used to quantitate interactions stronger than 1×10^{-9} M. For nitrocellulose-filter-binding assays, a 454-nucleotide RNA fragment containing the RRE was used as the specific ligand and either a 667-nucleotide nonspecific RNA transcript or a 440-nucleotide complementary antisense RNA transcript were used for comparison. Under standard equilibrium binding conditions, Rev bound in a concentration-dependent manner with either the RRE-containing

FIG. 3 Binding of Rev to the RRE-containing RNA, antisense RNA or non-specific RNA species by nitrocellulose-filter-binding assay. The RNA-binding assay used was a variation of the nitrocellulose-filter-binding assay described by Riggs *et al.*¹⁸. Typically, RNA fragments were added to PBS buffer supplemented with 50 $\mu\text{g ml}^{-1}$ BSA (binding buffer). To all samples, 10 U RNasin were added to inhibit RNase degradation. To insure that Rev-RNA binding would reach equilibrium, Rev was always the final addition to the RNA-containing samples and allowed to incubate for 15 min at room temperature. After incubation, the 0.5-ml samples were filtered at a constant rate (0.05 ml s^{-1}) through 25-mm 0.45- μm pore-size nitrocellulose filter disks (Millipore). Filters were dried for 15 min at 45°C and the adsorbed radioactivity was determined by scintillation counting. *a*, Direct binding of Rev to the 454-base RRE-containing RNA or 440-base antisense RNA fragments. The concentration of each ^{32}P -labelled RNA fragment was estimated at 2×10^{-11} M for each assay. Rev concentrations were varied from 8.6×10^{-11} M– 8.6×10^{-9} M in each assay. K_d is defined as the concentration of protein at 50% fractional saturation. K_d was also determined from the slope of a double reciprocal plot of $1/\text{fractional saturation}$ versus $1/\text{Rev concentration}$. *b*, Direct binding of Rev to ^{32}P -labelled 454-base RRE-containing RNA or ^{32}P -labelled 440-base antisense RNA in the presence of 1.45×10^{-9} M base concentration of unlabelled MS2 nonspecific viral RNA. Rev concentrations were identical to those used in *a*. *c*, Stoichiometry of the Rev-RRE-containing RNA interaction. Various concentrations of Rev were added to samples containing ^{32}P -labelled 454-base RRE-containing RNA (10,000 c.p.m. per 1.1×10^{-8} M RRE) in PBS buffer supplemented with 50 $\mu\text{g ml}^{-1}$ BSA. To determine the stoichiometry of binding it was critical that the concentration of ligand (RRE) was greater than the K_d . Rev concentrations were varied from 2.3×10^{-9} M– 4.6×10^{-7} M. Each 0.5-ml sample contained 10 U RNasin and 7.6×10^{-4} M base concentration unlabelled tRNA to remove any nonspecific component of the binding interaction. Samples were incubated for 15 min at 23°C and processed as described above. 454-base RRE, $\bullet$; 440-base antisense RNA, $\blacksquare$.

RNA species or nonspecific RNA (Fig. 3a). Equilibrium dissociation constants (K_d) determined from these experiments show that Rev bound the RRE-containing 454-mer RNA with a K_d of $\sim 3 \times 10^{-10}$ M. The binding constant was unaffected by the presence of 2.5×10^{-8} M unlabelled nonspecific RNA in the binding reaction, confirming the high specificity of Rev for the RRE-containing ligands (Fig. 3b). Direct binding of Rev to antisense RNA was examined in the absence of unlabelled nonspecific RNA. Under these conditions, the measured equilibrium binding constant for antisense RNA was about fourfold weaker than the binding constant for the specific interaction. But in the presence of 2.5×10^{-8} M nonspecific RNA, no binding of the 454-mer antisense RNA could be observed at any of the Rev concentrations studied. Similar binding results were obtained when the labelled ligand was the 667-mer nonspecific RNA fragment (data not shown). The differential effect of unlabelled competitor RNA in the binding reaction suggests that the specific Rev-RRE interaction is substantially more stable than the Rev-nonspecific RNA interaction. The sigmoidal nature of the nonspecific RNA binding curve also suggests that a significant amount of protein-protein interactions exists. Cooperativity in the Rev-nonspecific RNA interaction could explain the unusually low equilibrium K_d observed in these direct binding assays.

The ability of Rev to interact with the RRE-containing RNA fragment was inhibited by anti-Rev antiserum but not pre-immune serum (data not shown). The polyclonal anti-Rev antiserum was initially generated from Rev purified from insect cells infected with a baculovirus expressing Rev. It is therefore unlikely that any contaminating *E. coli* proteins in the Rev-containing samples will be recognized by the antiserum. When Rev protein was added to samples already containing anti-Rev antiserum and the Rev-responsive element, no binding was detected. But Rev added to RRE in the presence of preimmune serum was fully active in the assay, confirming that high-affinity RRE binding was due to direct interaction with Rev. Conversely, preformed Rev-RRE complexes were not disrupted by addition of the anti-Rev antiserum. These preformed Rev-RRE complexes were still recognized by anti-Rev antiserum, because addition of immobilized protein A specifically precipitates Rev-RRE complexes in the presence of immune sera (data not shown).

Various concentrations of Rev protein were incubated with 1×10^{-8} M RRE-containing RNA fragment of known specific activity. Under these binding conditions, at low Rev concentrations, all of the added Rev will be bound to the RRE with virtually no free protein in solution. Saturation of the RRE at high concentrations of Rev was measured (Fig. 3c). The stoichiometry of the interaction was estimated from the ratio of the Rev and RRE concentrations at the saturation breakpoint to be about eight Rev molecules per RRE. Although a portion of the Rev in solution may be inactive, the results are in general agreement with the multiple banding patterns observed in the gel shift assay. Equivalent stoichiometry results were obtained in the presence of excess unlabelled nonspecific RNA.

Because of the apparent tendency of Rev to self-associate to RNA fragments, an indirect competition assay was devised to assess the relative affinities of Rev for various nucleic-acid species (Fig. 4). Under equilibrium binding conditions, excess Rev was incubated with labelled RNA species in the presence of various concentrations of unlabelled competing RNA fragments. When the labelled RNA species was the 667mer nonspecific fragment, $\sim 2.3 \times 10^{-7}$ M base concentration of unlabelled MS2 viral nonspecific RNA was required for 50% inhibition of binding of the labelled RNA fragment by Rev (Fig. 4a). When the labelled 454-mer RRE-containing RNA was mixed with the unlabelled MS2 nonspecific RNA species, $\sim 3.6 \times 10^{-4}$ M base concentration of the unlabelled RNA species was required to achieve 50% inhibition of Rev binding. This result demonstrates that the affinities of specific and nonspecific RNA

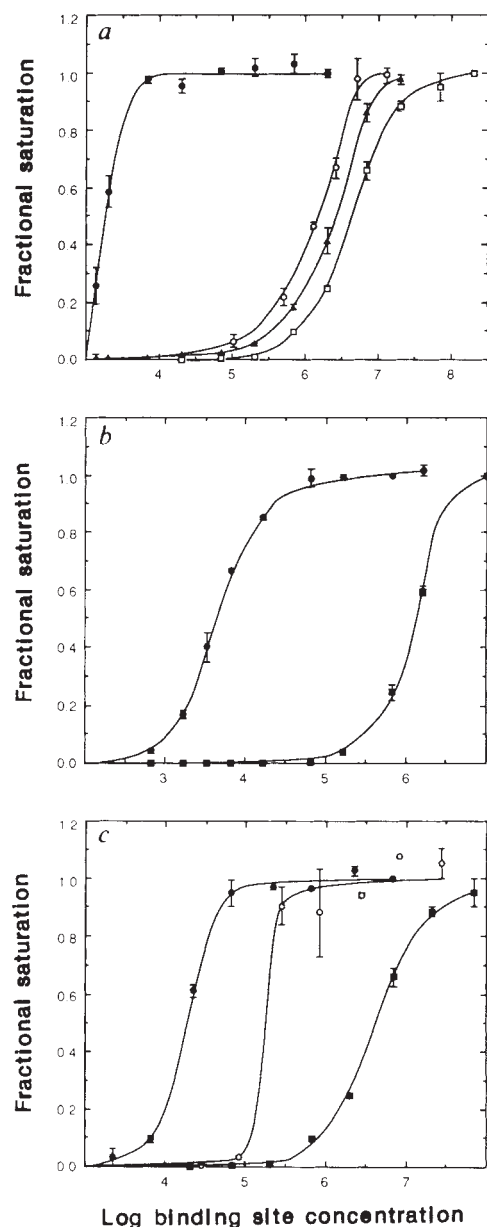


FIG. 4 Competition of Rev binding to ^{32}P -labelled specific and nonspecific RNA fragments by various unlabelled nucleic-acid species. Rev (8.6×10^{-9} M) was added to samples containing $\sim 2 \times 10^{-11}$ M ^{32}P -labelled RNA fragments and various concentrations of unlabelled competitor nucleic acid and incubated for 15 min at 23°C and processed as described in Fig. 3. The fractional saturation was defined as the ratio of radioactivity retained on the nitrocellulose filter, at a given competitor concentration, to the measured radioactivity in the absence of unlabelled competitor. Binding curves were compared at 50% fractional saturation (inhibition of binding) to determine relative binding affinities. a, Competition of Rev binding to: ^{32}P -labelled 454-base RRE-containing RNA by unlabelled 454-base RRE-containing RNA ($\circ$), or by unlabelled nonspecific MS2 viral RNA ($\bullet$); ^{32}P -labelled 667-base nonspecific RNA by unlabelled nonspecific MS2 viral RNA ($\blacktriangle$); and ^{32}P -labelled 440-base antisense RNA by unlabelled nonspecific MS2 viral RNA ($\square$). b, Binding of Rev to ^{32}P -labelled sense ($\bullet$) or antisense ($\blacksquare$) RNA fragment in the presence of various amounts of unlabelled tRNA. c, Competition of Rev binding to ^{32}P -labelled nonspecific RNA by various amounts of unlabelled nonspecific single-stranded DNA ($\circ$), nonspecific double-stranded DNA ($\bullet$) or nonspecific RNA ($\blacksquare$).

molecules for the binding of Rev actually differ by a factor of 1,600. Under physiological conditions, such a large difference in Rev affinity for specific and nonspecific RNA molecules would be necessary for Rev to successfully distinguish between the few mRNA molecules that contain the RRE and the majority of RNA fragments that do not.

Competition binding assays, performed using the 454-mer sense and 440-mer antisense RNA fragments as the labelled ligands, showed that the antisense RNA possessed predominantly nonspecific Rev-binding characteristics (Fig. 4a). The binding of Rev to either of these two RNA species was competed with various concentrations of unlabelled MS2 viral nonspecific RNA. For the antisense fragment, 50% inhibition was observed at $\sim 4.5 \times 10^{-7}$ M base concentration of unlabelled RNA. The concentration of unlabelled MS2 RNA required to obtain 50% inhibition of the binding of Rev to the labelled sense RRE-containing RNA fragment was $\sim 5.5 \times 10^{-4}$ M. Rev bound with 1,200-fold higher affinity to the sense (RRE-containing) RNA than it did to antisense RNA species, indicating that the nucleotide sequence rather than conformation is the predominant determinant for the specificity of Rev. On the basis of computer simulations of RNA folding patterns, however, the sense and antisense RNA species would not be expected to fold in an equivalent manner, and therefore the specificity of Rev due to RNA conformational differences cannot be completely dismissed.

To examine the influence of secondary structure on the binding of Rev to the RRE, unlabelled tRNA was also used as the competing ligand (Fig. 4b). With the antisense RNA as the labelled species, the tRNA base concentration required for 50% inhibition was $\sim 7 \times 10^{-7}$ M. This value is similar to the concentration of MS2 RNA required for 50% inhibition of Rev binding, and again demonstrates that antisense RRE is functionally equivalent to nonspecific RNA with respect to the binding of Rev. But to compete with the sense RRE-containing RNA, as much as 2.5×10^{-4} M tRNA base concentration was needed for 50% inhibition. The fourfold difference in relative affinity when tRNA was used as the competing species rather than MS2 RNA could be attributable to common structural motifs in tRNA and RRE such as those of double-stranded character and stem-loop structures.

Single-stranded and double-stranded DNA species competed with labelled nonspecific RNA for the binding of Rev only at high concentrations (Fig. 4c). The 667-mer nonspecific RNA fragment was used as the labelled ligand for each competition curve. Single-stranded DNA and double-stranded DNA possessed 30-fold and 250-fold lower affinities, respectively, for Rev binding than did nonspecific RNA. Single-stranded DNA containing the RRE sequence bound Rev with the same relative affinity as nonspecific single-stranded DNA in the filter assay (data not shown). The relative difference in affinity between Rev-RRE binding and Rev-dsDNA binding is $\sim 250,000$ -fold.

Specific binding of Rev to the RRE *in vitro* is thus demonstrated by both gel-shift and nitrocellulose-filter-binding assays. Nitrocellulose-filter binding was found to be a suitable method for both direct- and competition-binding experiments. An apparent K_d of 0.3 nM for the Rev-RRE interaction was measured by direct methods, and the interaction was shown to be about 1,600-fold tighter than the binding of Rev to nonspecific RNA or to antisense RNA by competition assays. The quantitative binding data indicate that multiple Rev molecules bind to the RRE. Whether this binding is physiologically relevant as is the case with some nucleic acid-binding proteins (such as SV40 large T antigen)¹⁴ remains to be determined. Multiple Rev proteins could be necessary to transport RRE-containing mRNA directly or could be required to alter the nucleic-acid structure to facilitate interaction with other RNA transport factors. Previously observed transdominant Rev mutant proteins¹² could possess wild-type RRE-binding activities but a decreased ability

to form protein-protein contacts with either additional Rev molecules or other transport factors.

Such *in vitro* assays will allow the quantitative biochemical analysis of Rev protein mutants¹² and RRE mutants. By this means, a correlation might be established between aspects of RRE binding *in vitro* and Rev-mediated gene expression leading to a more detailed understanding of the molecular mechanism of Rev protein function. In addition, the filter-binding assay could be a valuable tool in screening potential antiviral compounds capable of disrupting the specific Rev-RRE interaction. *Note added in proof:* Protein-dependent retardation of the electrophoretic mobility of RRE RNA by crude extracts from *E. coli* expressing recombinant Rev protein has recently been reported (Zapp, M. L. and Green, M. R. *Nature* **342**, 714-716 (1989)). □

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Conservation between yeast and man of a protein associated with U5 small nuclear ribonucleoprotein

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THE process of nuclear pre-messenger RNA splicing is similar in *Saccharomyces cerevisiae* and metazoan cells in that the two-step mechanism is identical and the reaction occurs in a large ribonucleoprotein complex, the spliceosome¹. Little is known, however, about the degree of conservation of splicing factors other than of the small nuclear RNAs (snRNAs). Yeast counterparts of the metazoan spliceosomal snRNAs (U1, U2, U4, U5 and U6) have been identified² but, with the exception of U6, the yeast snRNAs are larger and sequence similarity is limited to short regions. By using antibodies against the yeast PRP8 protein, a pre-mRNA splicing factor of relative molecular mass 280,000 (*M*_r 280K) stably associated with U5 small nuclear ribonucleoproteins (snRNPs)^{3,4}, we have now identified an immunologically related

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FIG. 1 *a*, Physical map of the *PRP8* gene. The arrow shows the position and orientation of the *PRP8* coding sequence relative to restriction sites used in the construction of fusions. Brackets show the regions that were fused to *lacZ* or *trpE* to produce fusion proteins. The number of amino acids (aa) specific to *PRP8* in each fusion is indicated. ORF, open-reading frame. *b*, *c*, Detection of a large HeLa cell protein immunologically related to *PRP8*. Aliquots of HeLa cell nuclear extract were analysed by western blotting, using as primary antibodies: unfractionated polyclonal antisera (*b*) or *PRP8*-specific antibodies affinity-purified by binding to the analogous TrpE-*PRP8* fusion protein (*c*). Primary antibodies used: (*b*) Anti-8.3 (lane 1), anti-8.1 (lane 2), anti-8.2 (lane 3), anti-8.4 (lane 4); (*c*), antibodies affinity-purified from anti-8.1 (lane 1), anti-8.2 (lane 2), anti-8.4 (lane 3) sera. The positions of protein M_r standards are indicated ($M_r \times 10^{-3}$).

METHODS. TrpE-*PRP8* fusions were produced using the plasmid pATH vectors¹¹ and the same regions of *PRP8* as were used to construct the corresponding *lacZ-PRP8* fusions, against which the rabbit antisera were raised (all as described in ref. 4). *PRP8*-specific antibodies were purified from the antisera by binding to the appropriate TrpE-*PRP8* fusion protein immobilized on nitrocellulose membrane followed by elution at low pH¹². HeLa cell nuclear extract was prepared according to ref. 13. Aliquots (6 μ l) of extract were fractionated in an 8.5% SDS-polyacrylamide gel, electroblotted to nitrocellulose, blocked, probed with primary antibodies (diluted 1,500-fold), and developed as described in ref. 4, except that all incubations and washes were performed in 1 $\times$ TBS, 0.2% (v/v) Tween 20.

protein in HeLa cell nuclear extracts. The HeLa cell protein has an M_r greater than 200K and is associated with purified 20S U5 snRNPs. This is the first report of phylogenetic conservation between yeast and man of a protein splicing factor.

Protein *PRP8* (formerly RNA8; refs 3,4) of *S. cerevisiae* is essential for nuclear pre-mRNA splicing *in vivo* and *in vitro* and is specifically associated with U5 snRNPs and U4-U5-U6 snRNP complexes⁴. HeLa cell nuclear extract was tested by immunoblotting for the presence of proteins that are recognized by antibodies raised against β -galactosidase-*PRP8* fusion proteins (anti-8.1-anti-8.4 antibodies, Fig. 1*a*). Several HeLa cell nuclear proteins were detected by antibodies in each of the sera (Fig. 1*b*) but, notably, a species with an $M_r > 200$ K was recognized by anti-8.1 (lane 2), anti-8.2 (lane 3) and anti-8.4 (lane 4), but not by anti-8.3 (lane 1) sera. Blots with the corresponding pre-immune sera were blank; blots using antibodies affinity-purified against β -galactosidase, however, demonstrated that most of the signals were due to spurious cross-reactions with antibodies against the β -galactosidase moiety of the fusion proteins (data not shown). Antibodies specific to the *PRP8* portions of the fusions were therefore affinity-purified against the corresponding TrpE-*PRP8* fusion proteins. *PRP8*-specific antibodies from anti-8.1, anti-8.2 and anti-8.4 sera detect the ~200 K species in HeLa cell nuclear extract (Fig. 1*c*), whereas those from anti-8.3 serum do not (data not shown). These antibodies do not recognize epitopes in common (data not shown) this protein therefore possesses epitopes present in three distinct regions of *PRP8*.

Immunoprecipitation of the ~200K HeLa cell protein by anti-*PRP8* antibodies could not be detected (assayed on immunoblots, data not shown). Anti-Sm antibodies, recognizing antigenic sites on snRNP-specific polypeptides and isolated from patient autoimmune-sera, were, however, able to precipitate a ~200K species which was detected by anti-8.4 antibodies on an immunoblot (Fig. 2*a*), but efficient precipitation required pre-incubation of the nuclear extract in the presence of ATP. As for the ~200K protein in the total nuclear extract, the anti-Sm-precipitable species was detected by anti-8.1, anti-8.2 and anti-8.4, but not by anti-8.3 antibodies (data not shown);

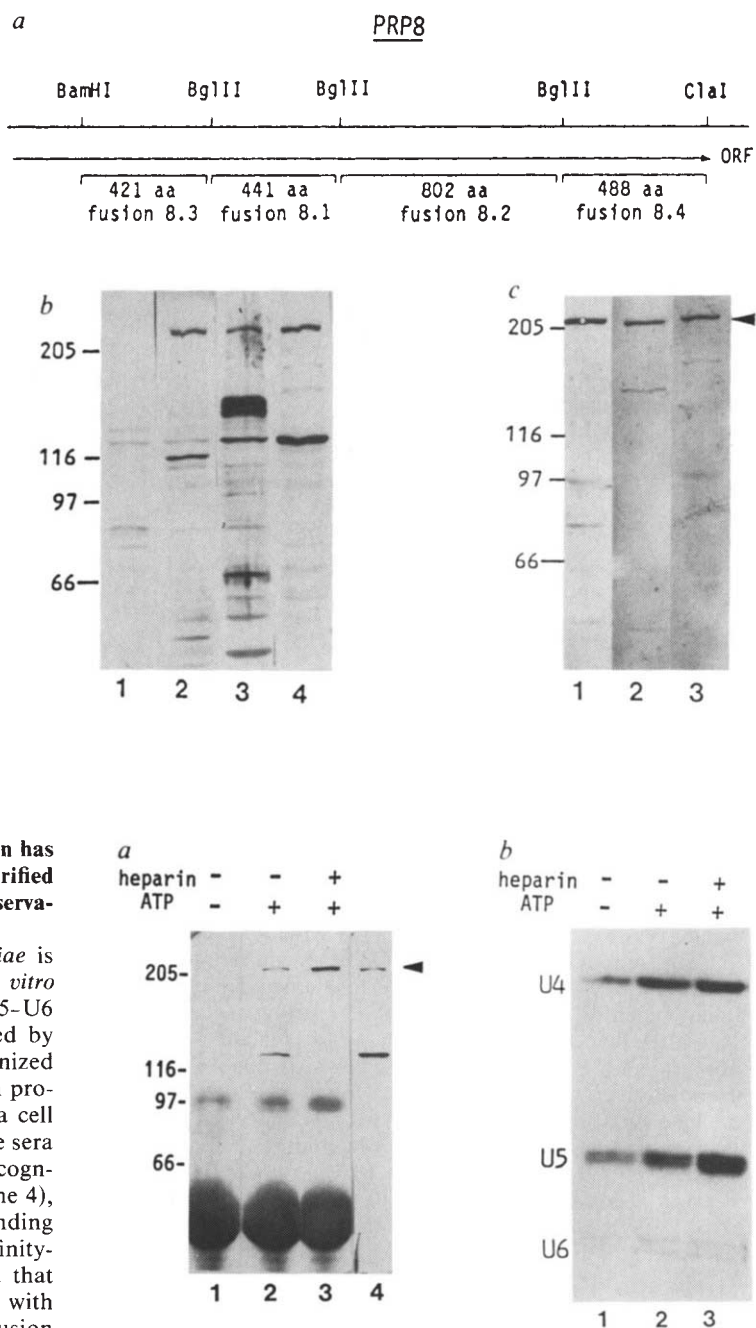


FIG. 2 The HeLa cell protein is present in anti-Sm-precipitates; the effect of ATP and heparin on immunoprecipitation by anti-Sm serum of the HeLa protein (*a*) and snRNAs (*b*). HeLa cell nuclear extract was incubated in the absence (lane 1) or presence (lanes 2 and 3) of 1.6 mM ATP, followed by incubation in the absence (lanes 1 & 2) or presence (lane 3) of heparin (5 mg ml⁻¹), and immunoprecipitated with anti-Sm antibodies. The precipitates were analysed by western blotting (*a*) for the presence of protein which cross-reacts with anti-8.4 antibodies, and by northern blotting (*b*) for the presence of U4, U5 and U6 snRNAs. *a*, Lane 4 shows a blot of 6 μ l untreated HeLa cell nuclear extract; the positions of protein M_r standards are indicated ($M_r \times 10^{-3}$).

METHODS. Aliquots (15 μ l) of HeLa cell nuclear extract were incubated for 40 min at 30 °C under splicing reaction conditions¹⁴ but without substrate RNA, and with or without 1.6 mM ATP as indicated (total volume, 30 μ l), then 3 μ l heparin (50 mg ml⁻¹; lane 3) or 3 μ l water (lanes 1 and 2) were added, and incubations continued for 5 min. Anti-Sm precipitates were obtained by incubation with human anti-Sm antibodies bound to protein A-Sepharose, and the precipitates were analysed by western blotting (*a*) as in Fig. 1, using anti-8.4 serum, or by extracting RNA from the immune complexes, and fractionating, blotting and probing it as described in ref. 4 with oligonucleotide probes specific for HeLa cell U4, U5 and U6 snRNAs (*b*).

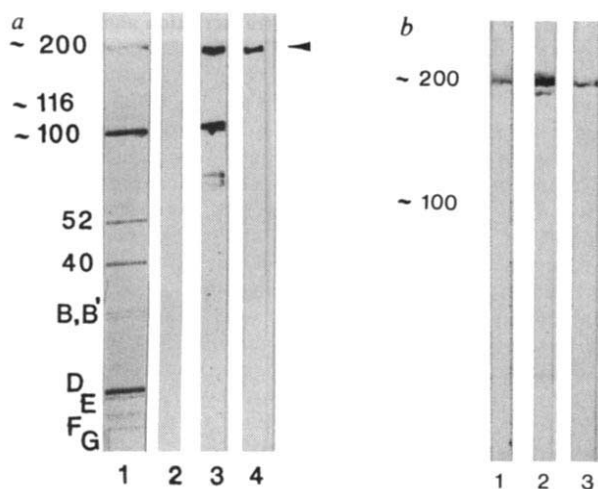


FIG. 3 Detection of the cross-reacting protein in purified HeLa cell U5 snRNPs. *a*, Proteins from purified HeLa cell 20S U5 snRNPs were fractionated by electrophoresis in a 4–20% gradient SDS-polyacrylamide gel and analysed by western blotting using pre-immune 8.2 serum as primary antibody (lane 2), immune anti-8.2 serum (lane 3), and anti-PRP8 antibodies affinity-purified from anti-8.2 serum (lane 4). The total protein content of the U5 snRNPs was visualized by staining with Coomassie blue (lane 1); proteins B, B', D, E, F and G which are common to the abundant U snRNPs are indicated; proteins specific to the U5 snRNP are identified by their apparent M_r s ($M_r \times 10^{-3}$). *b*, Proteins from purified HeLa cell 20S U5 snRNPs were fractionated by prolonged electrophoresis in an 8% SDS-polyacrylamide gel to resolve the doublet of bands corresponding to an $M_r > 200$ K, and analysed by western blotting using PRP8-specific antibodies affinity purified from anti-8.1 (lane 1), anti-8.2 (lane 2) or anti-8.4 (lane 3) sera. The approximate positions of size standards are indicated; proteins of lower M_r ran off the gel or were too diffuse to be detected.

METHODS. Purified 20S U5 snRNP fraction was prepared from HeLa splicing extract by anti-trimethylguanosine immunoaffinity chromatography, ion-exchange chromatography on a mono-Q column, followed by glycerol gradient centrifugation as described in ref. 5. Proteins were fractionated by SDS-PAGE and analysed by western blotting using unfractionated antiserum or PRP8-specific antibodies as in Fig. 1.

similar results were obtained using two unrelated anti-Sm sera). The same anti-Sm antibodies do not detect the ~200K protein by immunoblotting (data not shown), indicating that this protein is not directly recognized by these particular anti-Sm sera, although it does react weakly with certain other autoimmune sera⁵. It seems probable that the ~200K protein is anti-Sm-precipitable because of an association with an Sm antigen. Incubation of the nuclear extract in the presence of ATP also increases the anti-Sm-precipitability of U4, U5 and U6 snRNAs (Fig. 2*b*, compare lanes 1 and 2; U1 and U2 were not investigated). Incubation of both HeLa cell and yeast splicing extracts with ATP results in dissociation of high M_r multi-snRNP complexes, releasing free snRNPs, some of which recycle into other complexes^{4,6,7}. This re-organization of snRNPs probably explains the greater immunoprecipitability of yeast PRP8 protein and of yeast U5 snRNPs by anti-PRP8 antibodies in the presence of ATP⁴, and could also account for the observed effect of ATP on the anti-Sm precipitation of HeLa cell snRNPs and of the HeLa cell ~200K protein.

Incubation of HeLa cell nuclear extract with heparin after incubation with ATP results in a further increase in precipitation of the M_r ~200K protein and of the U4 and U5 snRNAs by anti-Sm-antibodies (Figs. 2*a, b*, lane 3). The effect of heparin, as assayed on non-denaturing gels, is to disrupt multi-snRNP complexes, resulting in the appearance of faster-migrating snRNA-containing particles, presumably free snRNPs (ref. 8 and G.J.A. unpublished results). These data indicate a possible association of the ~200K protein with an Sm antigen in snRNPs and indeed, the association of this protein with snRNPs is also indicated by its precipitation (less reproducibly) with antibodies against the trimethylguanosine cap specific to snRNAs (data not shown).

If the ~200K species is a snRNP protein, it could be associated with the U5 snRNP, as is PRP8 in yeast. Bach *et al.*⁵ have purified HeLa cell 20S U5 snRNPs that are functional in an *in vitro* complementation assay⁹. In addition to the seven proteins present in each of the main snRNPs U1, U2, U4–U6 and U5, HeLa cell 20S U5 snRNPs contain several unique proteins, including two with apparent M_r s in excess of 200K. To investigate whether either of these proteins corresponds to the species detected by anti-PRP8 antibodies, the protein content of purified HeLa cell 20S U5 snRNPs was analysed by immunoblotting (Fig. 3*a*). When probed with anti-8.2 antibodies (lane 3), signals at positions corresponding to M_r s of ~100K and >200K were observed. With affinity-purified anti-8.2 antibodies, however, only the slower migrating signal was observed (lane 4). This

indicates that the ~200K protein that is immunologically related to PRP8 is one of the proteins specifically associated with U5 snRNPs. In an experiment in which the signal corresponding to an $M_r > 200$ K was resolved as a doublet (Fig. 3*b*), the larger of the two species was most strongly detected by affinity-purified anti-8.1 (lane 1), anti-8.2 (lane 2) and anti-8.4 (lane 3) antibodies. It is not clear whether the smaller of the two ~200K species is derived from the larger, or represents a distinct, immunologically cross-reacting protein.

The functional significance of the ~200K HeLa cell protein in RNA splicing is further indicated by its presence in affinity-purified spliceosomes¹⁰, and by evidence that it becomes cross-linked to pre-mRNA in ultraviolet-irradiated HeLa cell spliceosomes (M. Garcia-Blanco, G.J.A., J.D.B. and P. Sharp, manuscript in preparation).

In *Drosophila melanogaster* whole-cell extract, a protein with an $M_r > 200$ K has been identified which shows the same pattern of recognition by the various anti-PRP8 sera as does the HeLa cell ~200K species (G.J.A., J.D.B. and D. Finnegan, unpublished results). Work is underway to analyse this protein and to investigate its possible relationship to splicing in *Drosophila*. It seems therefore that this protein is conserved across a wide phylogenetic spectrum, with respect to its large size, to several widely spaced epitopes, and to its association with U5 snRNPs. Such conservation, the first reported for a protein splicing factor, indicates a critical role for this species in the pre-mRNA splicing process. □

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Identification of a widespread nuclear actin binding protein

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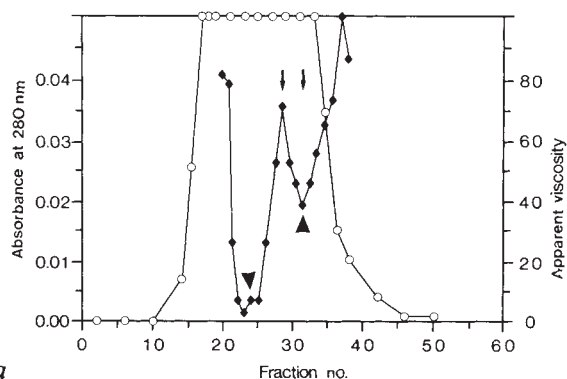
THE many different cellular functions so far shown to involve actin and to be regulated by specific actin binding proteins are located primarily, if not exclusively, in the cytoplasm¹⁻³. Actin is also found in the nucleus of various cells⁴⁻⁷, but because of the problems of cell fractionation the significance of nuclear actin has remained unclear. The large amphibian oocyte nucleus (germinal vesicle), however, can be isolated manually with little cytoplasmic contamination. This nucleus contains high concentrations (4–6 mg ml⁻¹) of mostly soluble, although polymerization-competent β - and

γ -actin⁸⁻¹⁴, which exists in a nucleocytoplasmic exchange pool¹⁵. The findings that drastic effects on transcription and chromosome morphology are caused by the injection of actin antibodies or actin binding proteins into germinal vesicles^{16,17}, and that a factor required for accurate transcription by RNA polymerase II is actin¹⁸, suggest that nuclear actin is involved in specific nuclear functions. We have recently identified^{19,20} two main components in *Xenopus laevis* oocytes with actin binding activities; one of these activities is Ca²⁺-dependent, is located predominantly, if not exclusively, in the cytoplasm and is attributable to gelsolin. Here we report that the second component, having a Ca²⁺-independent activity, is a heterodimeric actin binding protein; this protein is markedly enriched in the nuclei of oocytes and somatic cells of amphibia, but also occurs in nuclei of other vertebrate cells.

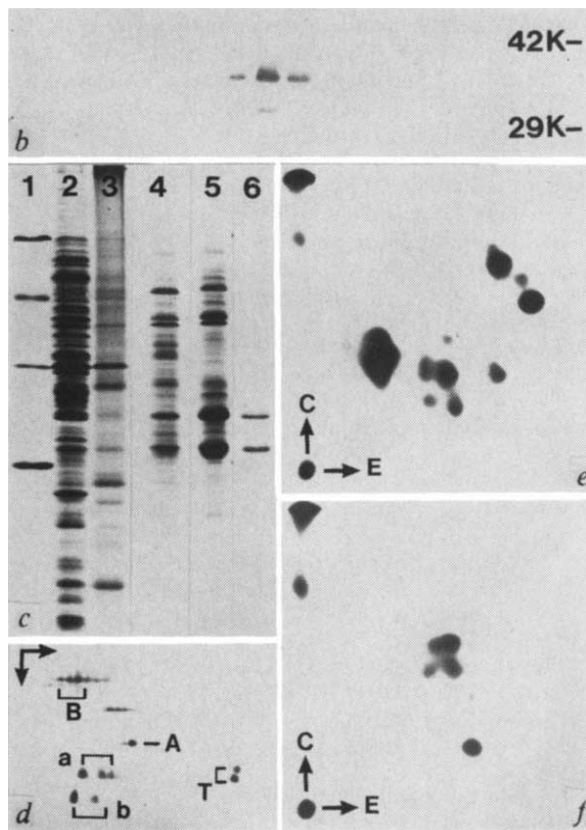
When soluble proteins from *X. laevis* oocytes or whole ovaries, as recovered from supernatant fractions after high-speed centrifugation¹⁹⁻²² were fractionated directly¹⁹ or after pre-fractionation by ion-exchange chromatography²⁰, and the fractions obtained assayed viscosimetrically for proteins interfering with the *in vitro* polymerization of actin, two main peaks were noted

FIG. 1 Fractionation of soluble proteins from *X. laevis* ovaries and characterization of the nuclear ABP. **a**, Activities inhibitory to actin polymerization in gel filtration fractions: $\circ$, absorption at 280 nm; $\blacklozenge$, apparent relative viscosity. Downward arrows denote the positions of BSA, (left) and ovalbumin (right) analysed in parallel; the arrowheads point to the activity peaks I (left; fraction 24) and II (right; fractions 30–32). **b**, Autoradiograph showing immunoblot results on SDS-PAGE-separated proteins of fractions described in **a** after reaction with antibodies to the chain a of the ABP (M_r 35K) in lanes containing the peak II fractions. M_r values of reference proteins run in separate lanes are shown on the right (actin and carbonic anhydrase). **c**, Coomassie blue staining of polypeptides separated by SDS-PAGE: Lane 1, reference proteins (from top: β -galactosidase, BSA, actin, carbonic anhydrase); lane 2, total supernatant; lane 3, pooled peak fractions of DEAE-Fractogel column chromatography containing the activity inhibitory to actin polymerization^{19,20}; lane 4, pooled peak II fractions of subsequent gel filtration (as in **a**); lane 5, pooled fractions containing maximal activity after subsequent Mono-Q ion exchange chromatography; lane 6, purified active protein after further chromatography on hydroxylapatite (note near-stoichiometric amounts of two polypeptides of M_r 35K and 31K). **d**, Coomassie blue staining of proteins separated by 2-D gel electrophoresis using isoelectric focusing in the first dimension (horizontal arrow) and SDS-PAGE in the second (downward arrow), of an ABP-enriched fraction, with BSA (B), α -actin (A) and two tropomyosin chains (T) added as internal references. Note that the chains a and b of the ABP have three isoelectric variants. **e**, **f**, Autoradiograms showing tryptic peptide maps of the radioiodinated 2-D gel electrophoresis-separated chain a (**e**) and chain b (**f**) polypeptides (C, chromatography; E, electrophoresis).

METHODS. Soluble proteins from *X. laevis* ovaries were prepared as 100,000g $\times$ 1-h supernatant fractions^{19,20} using buffer K (ref. 19) but without MgCl₂. The extract was then diluted 4:1 by addition of 20 mM Tris-HCl, pH 7.4, 0.2 mM CaCl₂, 1 mM 2-mercaptoethanol and 0.02% NaN₃ to final concentrations of 67 mM KCl and 20 mM NaCl and was either directly fractionated by gel filtration on a Sephadex G150 column (Pharmacia)¹⁹ or enriched by anion exchange chromatography before gel filtration on a DEAE-Fractogel column (5 $\times$ 26 cm; Merck), equilibrated with 20 mM Tris-HCl (pH 7.4), 0.2 mM CaCl₂, 67 mM KCl, 13 mM NaCl, 1 mM 2-mercaptoethanol and 0.02% NaN₃ (ref. 20). Unbound proteins were removed by washing with 1 l buffer, and absorbed proteins were eluted with a gradient of 80–160 mM KCl (same buffer)²⁰. Fractions were assayed for their inhibition of polymerization of rabbit skeletal muscle actin by low and high shear viscosimetry as previously described^{19,20}. Proteins of fractions reducing low shear viscosity were collected by ammonium sulphate precipitation and centrifugation, suspended in buffer K and fractionated further on Sephadex G150 at 0.8 ml min⁻¹ (**a**). Proteins of pooled peak II fractions were collected, applied to a Mono-Q anion-exchanger, and adsorbed proteins eluted with a 100–200 mM KCl gradient²⁰. Proteins of active fractions were dialysed overnight against 10 mM potassium phosphate buffer (pH 6.8) and separated on a hydroxylapatite column (Biorad)²⁰. SDS-PAGE and 2-D gel electrophoresis as well as immunoblotting and peptide map analyses were as previously described¹⁹⁻²². Antibodies were prepared by injecting guinea pigs subcutaneously with 50 μ g of the purified protein with an equal volume of complete Freund's adjuvant, followed by a booster injection (incomplete adjuvant) 3 weeks later.



a



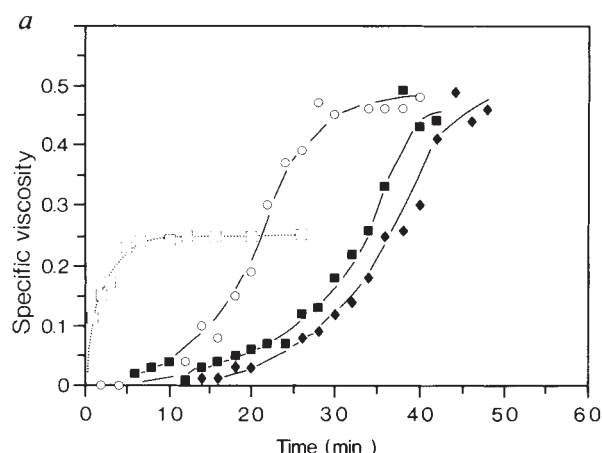
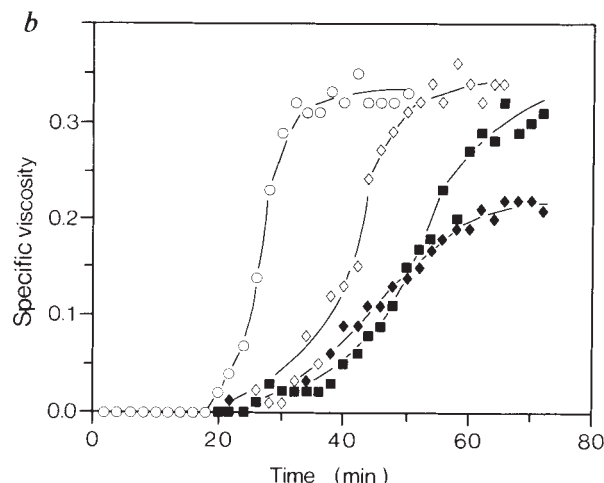


FIG. 2 Characteristics of the interference of the nuclear ABP of *X. laevis* oocytes with the *in vitro* polymerization of actin as determined by high shear viscosimetry. *a*, Polymerization of actin in the absence (○) and presence of 100,000g supernatant proteins from whole oocytes (□) and isolated nuclei (■, final protein concentration 0.1 mg ml⁻¹; ◆, 0.2 mg ml⁻¹). Note that in the presence of whole oocyte extract proteins (dotted line) the nucleation phase is shortened and the final viscosity reduced, reflecting the abundance of gelsolin in total extracts and in enucleated ooplasm¹⁹. By contrast, the addition of nuclear proteins (filled symbols) results in a lag of the nucleation phase, whereas the end viscosity is not considerably reduced



at these low concentrations. *b*, Effects of purified nuclear ABP on actin polymerization at different molar ratios of ABP: actin (○, control without ABP; ◇, ratio 1:5600; ■, 1:2800; ◆, 1:700). Note the high molar activity, resulting in an assembly lag period increasing with ABP concentration, whereas the end viscosity is only reduced at higher concentrations. METHODS. Nuclei from *X. laevis* stage VI oocytes were isolated, pelleted by centrifugation at 3,500 g for 10 min, and soluble proteins prepared as supernatant fraction¹⁹⁻²¹ (protein concentration 1–2 mg ml⁻¹). Purified nuclear ABP was obtained as described in Fig. 1. High shear viscosity of actin was determined in a Ostwald-type viscometer¹⁹ using 100 mM KCl (without MgCl₂) to start the reaction. The actin concentration was 0.5 mg ml⁻¹ (*a*) or 0.2 mg ml⁻¹ (*b*).

a

1 ATGAGGCTCTTTAATGATGTCGCGCTTTTACTCAACATGACAACCTGC
1 E V F N D V R L L L N N D N L

49 TCAGGGAAGGAGCAGCGCATGCTTTGCCAATATAACATGGATCAGT
16 L R E G A A H A F A Q Y N M D Q

97 TTACACCTGCTAAGATTGAAGGCTATGATGACCGAGTCTTATAACAG
32 F T P A K I E G Y D D Q V L I T

145 AGCATGGAGATCTTGGCAATAGCAGATTTCTGGATCCACGGAATCGAA
48 E H G D L G N S R F L D P R N R

193 TTACCTTTAAGTTTGATCACTTTAAGGAAAGAGCTAGTGATCCTCATC
64 I T F K F D H L R K E A S D P H

241 CTGACGACAGTGATGTGGCTTTAAATCCTGGAGAGATGCTTGTGACT
80 P D D S D V A L K S W R D A C D

289 TGGCCTTGCGGGCATATGTGAAGAACACTATCGAATGGTGTCTGCA
96 L A L R A Y V K E H Y P N G V C

337 CTGTTTATGGAAGCAATAGTGGACGCAACAAATAGTATCTTGTGTA
112 T V Y G K T I D G Q Q T I V S C

385 TTGAAAGCCACCAAGTTTCAGCCTAAGAACTTTTGAATGGTCGCTGGA
128 I E S H Q F Q P K N F W N G R W

433 GGTCTGAATGGAATTTACCATCTCGGGATCCACTGCTCAGTTGGTAG
144 R S E W K F T I S G S T A Q L V

481 GAGTTCTCAAGATTGAGTTTCATTACTATGAGGATGGAATGTTTCAGT
160 G V L K I Q Y H Y E D G N V Q

529 TAGTTAGCCACAAAGATGTGCGAAGATCCATAACAATCTCTGGTGAAG
176 L V S H K D V Q E S I T I S G E

577 CTCAAACGCCAAGGAATTTGTAAAAATATAGAGCAAGCAGAGAGTG
192 A Q T A K E F V K I I E Q A E S

625 ACTACGACAGTGCCTAAGCAGAACTATCAGACCATGTGACACACAA
208 D Y Q T A I S E N Y Q T M S D T

673 CTTTCAAAGCCTTACGCCGCGAGCTGCCAGTGACCCGCAACAGATCG
224 T F K A L R R Q L P V T R T K I

721 ACTGGAATAAAATCTCAGCTACAAAATTTGGCAAAGAGATGCAAAACG
240 D W N K I L S Y K I G K E M Q N

769 CTTGAGAACTTTATGGAGCAATGGCAAAGTTTGTGTGCAAGCAAA
256 A *

817 CTGAGAACTGTTTGGTCTTACAAATATACTATGTATACCTTATGTGT
865 AGGAGAGGCTCGTAACAAATGTATTGTTGGCCCCAACACAAATCAG
913 TTTTTTTTCAAAGGAGACAAAATATAGTGGTTGTGAAATGTGATATT
961 TTGCTAGGCGCTGCAGTCGTATTTCTAACTTGAAGTGCCGCGAGAACA
1009 ATCTTTTGTAGTTTTATATTATTTACCTTGAATCTCCAATTGAATTA
1057 CCGTATTTGTGAGCTGTGGCATATATCAAAATTTGCCAATAGAACATAT

b

MEVSDVRLPALLPSELNLSAGSTFREYNTSQMVQTSKGSALITKEGIISNNEYLDPNKQVITYDHI
** ***** 2 ** * * * 5 * * * 5 4 * 4 3 **64
NEVFNDRLLNNDLREGAAHAFAGYNMQFTPAKIEGVDQQLITEHGLDLSRFLDPNRNITFADF

KGEVTGERASGEIEQIEQYRAAFDEEATKYCNEYPNVGSVAVYGTKVSEGIKITYCISTCIYKPNAY
3 4 2 2 54 * * * 5 * * * * * 5 4 * 1 6 *
HLRKEADPHDDSDVALKSWRDACDLALRAYVKEHYNGVCTVYGTIDGGQITVSCIESHQGPKNFW

SGRWRSVMCTCFKPGSGNVTNKGQVNVHYFEDGNVQLNTVTQKQTTSPSADAGSTAVNAFKAIQKAEI
***** 1 5 * 5 5 ***** 1 * 1 * * * 5 * *
NGRWSEWKFITSGSTAQLV--GVKLQVHYEDGNVQLVSHKVGESITISGEAGTAKEFKIIEGAE

NLHTALDNNYSTMGDTTFKALRRALPINTKINWQVKNFKIANEL-NK*
** 5 ** * ***** **5 ***** 5 6** * 5 * *
DYGTATSENYGTMSDTTFKALRRQLPVTRTKIDWNKILSYKIGKEMGNA*

FIG. 3 Sequence of the cDNA clone λ ABPX1 encoding the chain *a* (*M_r* 35K) polypeptide of the nuclear ABP heterodimer of *X. laevis* and the deduced amino-acid sequence (*a*) compared with the amino-acid sequence (single-letter code) of the *M_r*-34K polypeptide subunit of the capping protein cap 32/34 of the slime mould *D. discoideum* (*b*). *a*, Nucleotide (top line) and deduced amino-acid (lower line) sequence of the cDNA clone λ ABPX1 isolated from a *X. laevis* oocyte expression library (amino-acid sequences directly determined from V8 protease fragments of purified nuclear ABP chain *a* polypeptide are underlined). Asterisk denotes stop codon. *b*, Alignment of the sequence shown in *a* with the corresponding sequence of the larger chain of the cap 32/34 protein of *D. discoideum*²⁴. Asterisks denote identical amino acids, except for the last which indicates the stop codon. Numbers indicate conservative exchanges (1, hydroxyamino acids; 2, acidic residues; 3, basic amino acids; 4, charged residues; 5, hydrophobic residues; 6, aromatic amino acids). Dashes denote omissions introduced for optimal alignment.

METHODS. A λ gt11 library prepared from poly(A)⁺ RNA of *X. laevis* ovary²² was screened with the antibodies to the nuclear ABP essentially as previously described^{19,22}. Positive clones selected were subcloned into M13mp15 and Bluescribe vector and identified by hybrid selection of oocyte messenger RNA and subsequent translation *in vitro*, which on SDS-PAGE revealed only the 35K polypeptide, and by northern blotting, which showed two mRNA bands of 1.8 and 1.9 kilobases, probably representing two alternative forms of polyadenylation. Procedures used in these experiments and for DNA sequencing were as previously described^{19,22}. For direct amino-acid sequence analyses, purified nuclear ABP was separated by SDS-PAGE, and the chain *a* dissected and digested in the gel section with V8 protease. The fragments were re-run on a SDS-PAGE gradient gel (15–22%), blotted electrophoretically onto Immobilon membranes (Millipore) and directly used for microsequencing in a pulsed liquid sequencer (model 477 A; Applied Biosystems).

(Fig. 1a). The activity of peak I is ascribed to gelsolin¹⁹, whereas the smaller peak II represented a Ca^{2+} -independent activity²⁰. During enrichment of the active component from pooled peak-II fractions by sequential application of Mono-Q anion exchange and hydroxyl apatite chromatography (Fig. 1a-c), we found that the activity interfering with actin polymerization was associated with a protein with a Stokes radius of 3.55 nm and an *S* value of 4.0 (as determined by sucrose gradient centrifugation), corresponding to an estimated apparent relative molecular mass of ~63,000 (*M_r* 63K). On SDS-PAGE this protein appeared as a doublet of polypeptides of *M_r* 35K (chain a) and *M_r* 31K (chain b) in a near-stoichiometric ratio (Fig. 1c). The two chains co-migrated on isoelectric focusing of the native protein, but on two-dimensional gel electrophoresis under denaturing conditions they were resolved into two components each appearing as a series of isoelectric variants (Fig. 1d), with main components at positions corresponding to pH 6.32 (chain a) and 6.36 (chain b), indicative of the presence of phosphorylated forms. Tryptic peptide maps of these spots showed that both chains were different (Fig. 1e, f). These results indicate that the protein is a heterodimeric actin binding protein (ABP). The protein was also found to be associated with the principal activity interfering with actin assembly in extracts of soluble proteins²¹ from isolated oocyte nuclei (Fig. 2a, b) in which gelsolin was greatly reduced or not detectable at all^{19,20}.

Apart from its independence of Ca^{2+} , the heterodimeric oocyte ABP displayed several other fundamental differences from the main cytoplasmic ABP, that is, gelsolin. For example, in 'high-shear viscosimetry' assays (Fig. 2a, b) the heterodimeric ABP considerably delayed the onset of filament formation at

very low concentrations (Fig. 2a, b), whereas a reduction of the 'end viscosity', that is, the final filament length, was noted only at elevated concentrations (Fig. 2b). These and other kinetic data²⁰ indicated that in the presence of this ABP the 'critical concentration' for actin assembly was greatly increased. When the severing of actin filaments was attempted, only a small effect was noted at relatively high concentrations²⁰, the significance of which remains doubtful.

Antibodies raised against the purified chains were used to isolate complementary DNA clones from a *X. laevis* oocyte λ gt11 expression library²². Positive clones were characterized by immunoblot reaction, by combined hybrid selection/*in vitro* translation, followed by gel electrophoretic analysis of the product, and by northern blot analysis (data not shown; Fig. 3). Nucleotide sequencing of the longest cDNA clone obtained, combined with direct amino-acid sequence analyses of proteolytic fragments from the PAGE-separated chain a (Fig. 1c, lane 6), showed that it contained the reading frame for most (~90%) of the polypeptide (Fig. 3a). The amino-acid sequence deduced showed no significant homology to any of the published sequences of other ABPs, except for the chain of *M_r* 34K of the heterodimeric F-actin 'capping protein' (cap32/34) of the slime mould *Dictyostelium discoideum*, particularly in the C-terminal 50 amino acids (Fig. 3b). The similarity between the *Xenopus* and the *Dictyostelium* proteins seemed even more

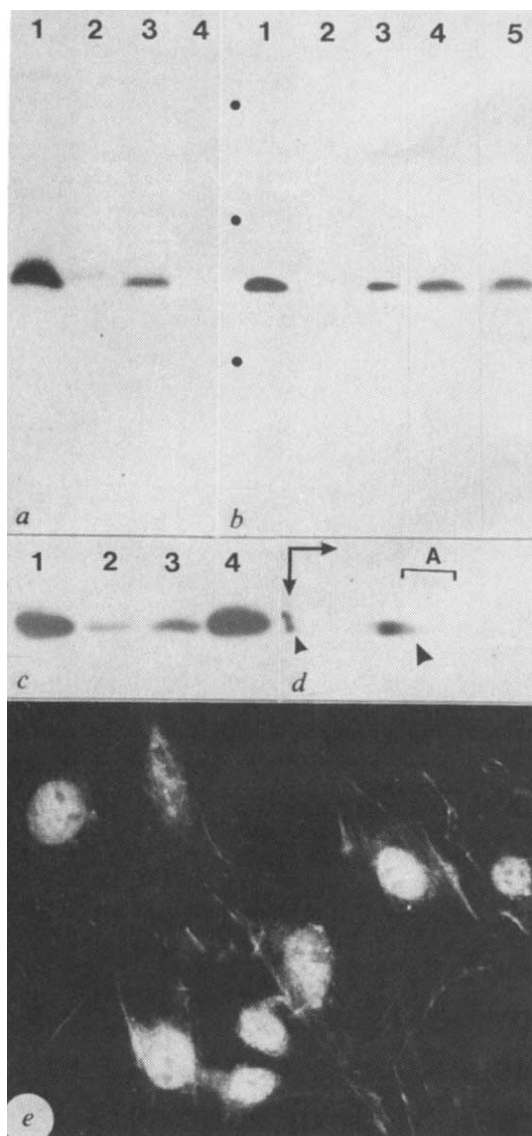


FIG. 4 Nuclear enrichment and widespread occurrence of the nuclear ABP in somatic cells of the same (*X. laevis*) and other species by immunoblot reaction (a-d) and immunofluorescence microscopy (e). a, Autoradiograph showing immunoblot reaction of soluble proteins (as in Fig. 1) separated by SDS-PAGE with the antibodies to the nuclear ABP 35K polypeptide from *X. laevis* and then with ¹²⁵I-labelled secondary (goat) antibodies to guinea pig immunoglobulin. Lane 1, proteins from whole oocytes; lane 2, total proteins of two manually enucleated ooplasm after fixation with trichloroacetic acid; lane 3, total proteins of sixteen manually isolated nuclei; lane 4, total proteins of two unfixed ooplasm. As the nuclear-cytoplasmic volume ratio in such oocytes is ~1:8 for the diffusion-accessible space (for discussion see ref. 11), the intensities of the reactions in lanes 2 and 3 are directly comparable. Note the drastic enrichment in the nucleus (lane 3). b, Autoradiograph showing the immunoblot reaction (same antibodies) with soluble proteins from different tissues of *X. laevis*: Lane 1, ovary; lane 2, negative control; lane 3, skin (epidermis and dermis); lane 4, liver; lane 5, brain. Note specific reaction of the ABP chain a in all tissues. Dots denote positions of *M_r* reference proteins: BSA, actin and carbonic anhydrase. c, Autoradiograph showing the immunoblot reaction with soluble proteins from *X. laevis* oocytes (lanes 1 and 4) and with total cell proteins of cultured *X. laevis* kidney epithelial cells of line XLKE-A6 (lane 2) and cultured bovine lens epithelial cells (lane 3). d, Autoradiograph showing co-electrophoresis of the chain a of the ABP from *X. laevis* oocytes and from XLKE-A6 cells after 2-D gel electrophoresis (symbols as in Fig. 1d) and immunoblotting (as in a-c). The large arrowhead denotes the ABP moiety that has entered the isoelectric focusing gel, the smaller arrowhead the moiety that has remained at the start. e, Immunofluorescence microscopy showing the reaction of the antibodies to the chain a of the heterodimeric ABP from *X. laevis* on a monolayer culture of bovine lens epithelial cells. Note intense but diffuse immunostaining of the entire nucleoplasm, leaving the nucleoli unstained, and some weak staining of actin filament cables and cell boundaries. Scale bar, 25 μ m.

METHODS. Nuclei and ooplasm of *X. laevis* oocytes fixed in 10% trichloroacetic acid were manually separated¹⁹. Small pieces of tissue or cell culture monolayers were either lysed directly in the buffer used for gel electrophoresis or were homogenized to prepare supernatant protein fractions^{19,20,29}. Conditions for gel electrophoresis and immunoblotting were as described in Fig. 1. For immunofluorescence microscopy of cells grown on cover slips, precautions were taken to minimize losses of soluble proteins. Therefore, the normal immunolocalization protocol^{9,29} was modified so that all buffers contained additional 1 mM MgCl_2 and the individual incubations were kept very short (10 or 15 min).

pronounced in their patterns of α -helical domains predicted by the method of Garnier *et al.*²⁵ (not shown).

The heterodimeric ABP is a soluble protein and thus can be rapidly lost by diffusion during isolation of nuclei in aqueous solutions (compare, for example, refs 9 and 11). Therefore we used both unfixed and trichloroacetic acid (TCA)-fixed^{19,20} oocytes for the determination of this protein in manually isolated oocyte nuclei and in enucleated ooplasm by immunoblot reactions (Fig. 4a). The concentration of this protein in the nucleus was at least 12-fold higher than it was in the cytoplasm, as estimated from densitometry of autoradiograms.

The heterodimeric ABP is not restricted to *X. laevis* oocytes. Using the immunoblot technique on SDS-PAGE-separated proteins, we detected the heterodimer also in oocytes of other amphibian species as well as in different tissues and in cultured cells of *Xenopus* (Fig. 4b-d). The protein also occurred in various tissues and cultured cell lines of other animals (Fig. 4c), including mammals (such as rat kangaroo, mouse, rat, bovine and human). As localization of soluble proteins by immunofluorescence microscopy is difficult, special precautions had to be taken to prevent antigen extraction during cell membrane permeabilization and incubations⁹. But by using modifications of the usual technique we were able to localize this protein in a variety of tissues and cultured cells. Figure 4e presents an example of a bovine cell line, illustrating the enrichment of the heterodimeric ABP in the nucleus where it appears dispersed over the entire nucleoplasm.

The identification described here of the main nuclear ABP of the amphibian oocyte provides a candidate molecule for the regulation of the structural form (for example, gel-sol transformations) and of the biological functions of nuclear actin. Moreover, the detection of this ABP in various other kinds of cells and in a wide range of species indicates its widespread occurrence as a nuclear component. Its heterodimeric organization, specific effects on actin polymerization and sequence homology to the heterodimeric actin-capping protein of *Dictyostelium* suggest that the nuclear ABP is related to this slime mould protein as well as to the heterodimeric capping proteins of *Acanthamoeba*³, mammalian brain^{3,27} and chicken skeletal muscle (capZ36/32)²⁸, although the latter has been localized to sarcomeric Z-disks²⁹ (the antibodies used in the present study did not specifically immunostain Z-lines of muscle tissue). For none of these other probably related heterodimeric ABPs has a nuclear localization been reported. Although the ABP described here is enriched in the nucleus it is obvious that some of it also occurs in the cytoplasm, and in some cell cultures we have observed weak cytoplasmic immunostaining, also on actin fibrils and at cell boundaries (data not shown). But the significance of these weak reactions remains to be examined. The molecular probes to the heterodimeric ABP described here should help to elucidate the possible functions of this protein in the nucleus as well as in the cytoplasm.

Note added in proof: Since submission of this manuscript, we have observed that the complete sequence of the larger subunit of the avian capZ36/32 protein³⁰ is highly homologous to the chain α of the nuclear ABP of *Xenopus*. Also, a nuclear ABP of *Acanthamoeba* has been reported to be a homodimer of a polypeptide of M_r 34K and also to bind to DNA³¹. A relationship of this nuclear ABP to the *Xenopus* heterodimer described here cannot at present be excluded. □

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Telomeric DNA dimerizes by formation of guanine tetrads between hairpin loops

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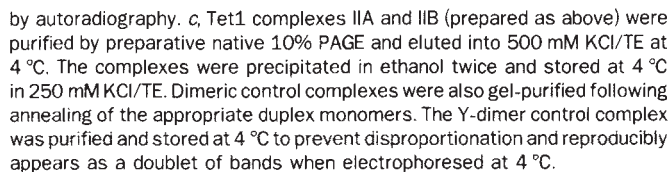
THE telomeric ends of eukaryotic chromosomes are composed of simple repeating sequences in which one DNA strand contains short tracts of guanine residues alternating with short tracts of A/T-rich sequences^{1,2}. The guanine-rich strand is always oriented in a 5'-3' direction towards the end of the chromosome and is extended to produce a 3' overhang of about two repeating units in species where the telomeric terminus is known^{3,4}. This overhang has been implicated in the formation of several unusual intra- and intermolecular DNA structures⁵⁻⁹, although none of these structures has been characterized fully. We now report that oligonucleotides encoding *Tetrahymena* telomeres dimerize to form stable complexes in solution. This salt-dependent dimerization is mediated entirely by the 3'-terminal telomeric overhang (TTGGGGTTGGGG) and produces complexes in which the N7 position of every guanine in the overhangs is chemically inaccessible. We therefore propose that telomeric DNA dimerizes by hydrogen bonding between two intramolecular hairpin loops⁵, to form anti-parallel quadruplexes containing cyclic guanine base tetrads. These novel hairpin dimers may be important in telomere association and recombination and could also provide a general mechanism for pairing two double helices in other recombinational processes.

Telomeres are essential for chromosome stability and also appear to mediate the association of chromosomes during meiosis and mitosis¹. It has been suggested by Sen and Gilbert that telomeric DNA sequences may associate to initiate the alignment of four sister chromatids by forming parallel guanine quadruplexes (termed G4 DNA)⁸. To investigate this hypothesis, we have examined the salt-dependent association of oligonucleotides Tet1 and G12 (shown in Fig. 1a). Tet1 consists of a double-helical section containing the (TTGGGG) repeating sequence found in telomeres from *Tetrahymena*, followed by a single-stranded 3'-terminal overhang of two repeats^{3,10}. The oligonucleotide G12 is identical except that the 3'-terminal overhang contains 12 guanines and might therefore be expected to form parallel G4 DNA more readily than the heteropolymeric Tet1 overhang. As shown in Fig. 1b (lanes 1-7), Tet1 slowly

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METHODS. *a* and *b*, Single-stranded oligonucleotides were synthesized by the phosphoramidite method and purified to homogeneity by preparative denaturing polyacrylamide gel electrophoresis (PAGE)³⁵. All electrophoresis was performed at the indicated percentages of 19:1 acrylamide:*bis*-acrylamide in 1 × TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0). The cytosine-rich oligonucleotide was radiolabelled at the 5' end with polynucleotide kinase³⁶ and annealed to each of the complementary oligonucleotides in 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Annealed duplexes were subsequently purified by preparative native 10% PAGE, excised, eluted into 1 × TE buffer, ethanol-precipitated twice from 150 mM NaCl, washed extensively with 7:3 ethanol:water, and diluted into the appropriate incubation buffer. Association incubations were performed for 48 h at 23 °C with oligonucleotide concentrations of 2×10^{-7} M (assuming $\epsilon = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$ per nucleotide; $462,000 \text{ M}^{-1} \text{ cm}^{-1}$ per oligonucleotide). Solutions were buffered with 1 × TE and contained the indicated NaCl concentrations. Tet1 complexes were analysed by nondenaturing 10% PAGE at 4 °C to prevent dissociation (16 h, 7.5 V cm^{-1}). Gels were dried and visualized



self-associates in a salt-dependent fashion to form two electrophoretically distinct complexes (IIA and IIB). In contrast, the control oligonucleotide G12 does not form any stable complexes under identical conditions (lanes 8–14). This surprising result suggests that the two pairs of thymidines in the 3'-terminal overhang play an important role in the self-association of Tet1 and that complexes IIA and IIB are not simply tetramers stabilized by parallel guanine quadruplexes (however, a putative tetramer of Tet1 is observed as a minor species when the annealing is performed at the relatively high concentration of 10^{-4} M oligonucleotide). Complexes analogous to IIA and IIB are also formed by the sequences characteristic of telomeres from *Oxytricha* which consist of double helical (T_4G_4) repeats and a single-stranded 3'-terminal ($T_4G_4T_4G_4$) overhang⁴ (data not shown).

At ambient temperature, complexes IIA and IIB dissociate under the low-ionic strength conditions of electrophoresis. These complexes are kinetically stable at low temperatures, however, and may therefore be purified by preparative gel electrophoresis at 4 °C. Both complexes are stabilized by potassium and may be stored intact in buffers containing more than 100 mM KCl, a result which is in good agreement with a previous report that among the common monovalent and divalent cations, potassium uniquely stabilizes associated telomeres from *Oxytricha nova*⁷. As shown in Fig. 1c, complexes IIA and IIB show electrophoretic mobilities that are intermediate between a continuous, dimeric control helix of 70 base pairs (bp) (I-dimer) and a Y-shaped control dimer of the same molecular weight (Y-dimer). This result suggests that both complexes are dimeric forms of Tet1 and very probably have branched configurations intermediate between those of the two controls.

Complexes IIA and IIB may be shown conclusively to be dimers in an experiment analogous to that described for proteins by Hope and Struhl¹¹. In this experiment, the stoichiometry of complexes IIA and IIB is analysed by co-annealing two telomeric oligonucleotides of different sizes and observing the number of intermediate complexes formed. A truncated telomeric oligonucleotide (Tet2) was created for this purpose by removing 8 base pairs from the non-telomeric end of Tet1 with the restriction enzyme, *Sma*1. As shown in Fig. 2a, Tet1 and Tet2 co-anneal to form the expected A and B pair of heterodimers (lane 6, designated Tet1/Tet2, A and Tet1/Tet2, B). Alternatively, the complexes IIA and IIB can be purified and each shown (Fig. 2b) to give rise to a single heterodimer (Tet1/Tet2) on partial digestion by *Sma*1 (which cleaves one of the two double-helical arms present in each complex). Further *Sma*1 cleavage (of the second arm) yields the Tet2/Tet2 homodimer of each type, A or B. These Tet2/Tet2 homodimers are not observable, however, when the ³²P label is localized at the 5' end of the G-rich strand because two *Sma*1 cleavages completely remove the label from the complex (Fig. 2b, lanes 3, 12). The assignment of complexes IIA and IIB as dimers of Tet1 is uniquely consistent with all of these data.

To study the nature of interactions within the dimeric complexes IIA and IIB, the accessibility of guanine N7 positions was probed using the reagents dimethylsulphate (DMS)¹² and diethylpyrocarbonate (DEP)¹³. As shown in Fig. 3, all eight of the 3'-terminal guanines in the oligonucleotides constituting each complex are protected from reaction with either DMS or DEP, whereas guanines within the complementary regions react strongly with DMS but not DEP (lanes 2, 3, 5, 6). An exception is the slight, but reproducible, DEP hyperactivity of the first (3') guanine in the duplex region of both IIA and IIB (lanes 5, 6). Control reactions performed on the monomeric Tet1 oligonucleotide under identical conditions show strong DMS reactivity at all guanines and preferential DEP reactivity with guanines in the single-stranded 3' overhang as expected (lanes 1, 4).

The chemical protection patterns of IIA and IIB indicate that dimerization is mediated exclusively by the guanine-rich telomeric overhangs of Tet1, with all guanines in the overhangs

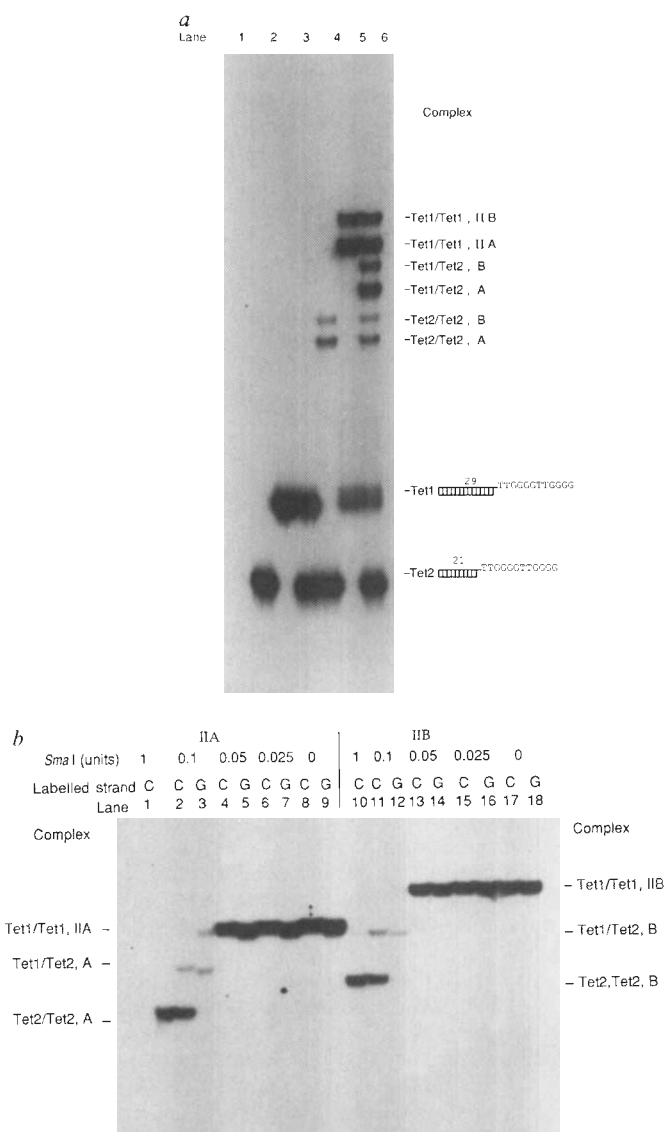


FIG. 2 Demonstrations that complexes IIA and IIB are dimers of Tet1. **a**, PAGE analysis of the dimeric complexes of Tet2 (lane 1, 4), Tet1 (lane 2, 5), and Tet1 plus Tet2 (lanes 3, 6). Oligonucleotides were incubated in 0 mM NaCl (lanes 1–3) or 500 mM NaCl (lanes 4–6). The resulting homo- and heterodimers are labelled with their composite oligonucleotides and with the designation A or B, in analogy to Fig. 1b. **b**, Identification of the individual intermediate species in **a** by PAGE analysis of the products formed by *Sma*1 digestion of purified complexes IIA (lanes 1–9) and IIB (lanes 10–18). Complexes were labelled at the 5' end of the cytosine-(C) or guanine-(G) rich strand and digested with the restriction enzyme *Sma*1 as indicated. Partial and complete digestion products are labelled as in **a**. **METHODS**. **a**, The oligonucleotide Tet2 was created by *Sma*1 digestion of Tet1 and was subsequently purified by preparative native PAGE. Tet1 and Tet2 were incubated alone or together in 1 × TE or 0.5 M NaCl/TE for 48 h at final oligonucleotide concentrations of 1×10^{-6} M. The resulting complexes were analysed by 8% PAGE (4 °C). **b**, Either strand of Tet1 was radiolabelled at the 5' end and complexes IIA and IIB were made and purified as described in the caption to Fig. 1. Each complex (~25 ng) was digested for 60 min at 23 °C with *Sma*1 in a digestion buffer of 50 mM KCl, 6 mM MgCl₂, 6 mM Tris/HCl, and 6 mM 2-mercaptoethanol, pH 8.0. The digestion products were analysed by native 10% PAGE.

involved. The DEP hyperactivity at the first guanine in the adjacent duplex may arise through partial disruption of this base pair when the dimeric structure is formed. Complete protection of all guanine N7 positions within the 3' overhang suggests the formation of cyclic guanine base tetrads, as previously proposed for poly(G) and also for G4 DNA (Fig. 4a)^{8,14,15}. The

postulation of a parallel G4-DNA structure⁸, however, is ruled out by the fact that IIA and IIB are dimers rather than tetramers. We propose instead that the single-strand overhangs of Tet1 form hairpin structures that dimerize to form antiparallel quadruplexes stabilized by guanine base tetrads (Fig. 4b). The presence of two thymines interrupting the runs of guanines (and shown above to promote dimerization) makes possible the folding back of the overhang into a hairpin.

In the course of model-building studies, we have observed two general rules for construction of stereochemically acceptable guanine quadruplexes that allow the incorporation of both parallel and antiparallel stands. First, as previously suggested, guanine quadruplexes must have some helical twist to allow the guanine tetrads to stack at 3.4 Å (ref. 14). X-ray diffraction patterns from oriented fibres of poly(G) confirm this 3.4 Å stacking and are consistent with an average helical repeat of 11.5 bases¹⁵. Second, the relative polarity of adjacent strands in the quadruplex mutually restricts their glycosidic conformations. Specifically, if adjacent strands are parallel, then the conformations of paired guanines between them must be the same (namely, *syn/syn* or *anti/anti*). Alternatively, if adjacent strands are antiparallel, then the conformations of paired guanines must be different (namely, *syn/anti* or *anti/syn*). These rules generate a number of distinct antiparallel quadruplexes that can be constructed from two TTGGGGTTGGGG strands and readily explain the observation of two structurally inequivalent Tet1 dimers (complexes IIA and IIB). Theoretically, a multiplicity of isomers is possible and this will be discussed in detail elsewhere. Indeed, preliminary investigations indicate that four dimeric forms of the simple single-stranded oligonucleotide TTGGGGTTGGGG (that is, the overhang alone) may be resolved electrophoretically, raising the possibility that the influence on electrophoretic mobility of the long duplex regions in complexes IIA and IIB may conceal subtle stereochemical differences in the region of Tet1 dimerization.

A reasonable mechanism for the formation of Tet1 dimers is through the annealing of precursor structures present in each overhang, possibly intramolecular hairpins stabilized by G-G Hoogsteen base pairs^{5,9}. Evidence for the existence of such hairpin structures is provided by the anomalously rapid electrophoretic mobility of the oligonucleotide TTGGGGTTGGGG at low temperature and low ionic strength⁵. Each G-G base pair in such a hairpin, however, is unsaturated in its hydrogen-bonding potential and has the capacity to form four more

hydrogen bonds along one edge. Furthermore, the spatial distribution of these hydrogen-bonding positions (N7-acceptor/O6-acceptor/N1-donor/N2-donor) is self-complementary and should therefore promote self-dimerization to form a guanine tetrad, provided the ionic strength is sufficient to overcome the phosphate-phosphate repulsions in a quadruplex.

Stacking of guanine tetrads into a continuous quadruplex creates cavities within the helix that are the correct size and geometry for potassium chelation. Figure 4c shows the cavity formed by the eight O6 atoms from two stacked guanine tetrads¹⁵. The average K-O bond length within this cavity (2.80 Å) falls nicely within the range observed for a large number of octacoordinate polyether potassium complexes and also agrees well with the K-O bond length calculated by summing the appropriate radii (2.78 Å)¹⁶. In contrast, the cavity is significantly larger than is typical for Na⁺ chelation (calculated Na-O, 2.40 Å), thus providing a rationale for the selective stability imparted by K⁺ binding to these structures. Binding of alkali metal and ammonium cations within the axial channel of quadruplex poly(I) has been reported previously, although selective binding of K⁺ by poly(I) is not observed^{14,17}. The unique potassium selectivity of guanine quadruplexes may reflect the greater rigidity of the chelation cavity resulting from the additional four N7...HN2 hydrogen bonds in a guanine tetrad versus an inosine tetrad.

In summary, the proposed hairpin dimers composed of antiparallel guanine quadruplexes satisfy the restrictive constraint that every guanine N7 atom within the region of dimerization be chemically inaccessible. This model also explains the generation of inequivalent Tet1 dimers and the unique stability imparted to these dimers by potassium. It appears that the central two pairs of thymines in the telomeric overhangs serve to break up continuous guanine stacking interactions and allow the single strands to fold back upon themselves by creating loops. The 5' thymines may act as spacers to reduce steric clash between the quadruplex and adjacent duplex regions and between the two duplexes themselves. Consistent with the postulated roles for these thymines, we find that both pairs of thymines in the two dimeric structures are accessible to chemical attack by OsO₄, whereas thymines in the double-helical regions are not (data not shown). We would not rule out the additional possibility, however, that the thymines may also stabilize the structure by hydrogen bonding between themselves in the loops (compare with ref. 18). Telomeric DNA sequences in general appear to

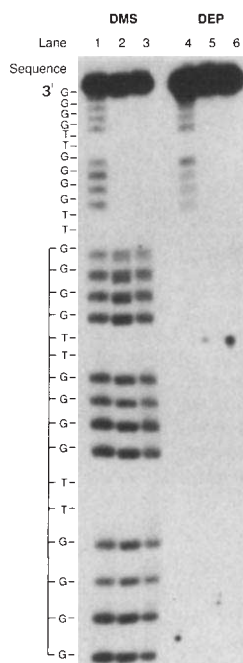


FIG. 3 Chemical accessibility of guanines in telomeric dimers. The oligonucleotide Tet1 (lanes 1, 4), and its purified complexes IIA (lanes 2, 5) and IIB (lanes 3, 6) were modified with the alkylating agents DMS (lanes 1–3) and DEP (lanes 4–6). Sites of alkylation were revealed by denaturing PAGE analysis following piperidine depurination and strand cleavage.

METHODS. Tet1, and its complexes IIA or IIB, labelled at the 5' end of the G-rich strand, were incubated for either 2 min in 0.5% DMS or 30 min in 5% DEP. DNA concentrations were 5 µg ml⁻¹ in 100 mM KCl/TE. The alkylated DNA was depurinated and cleaved in 10% piperidine at 90 °C (refs 12, 35). Complexes were denatured by heating to 90 °C in 80% formamide for 3 min before analysis by denaturing 20% PAGE (7 M urea).

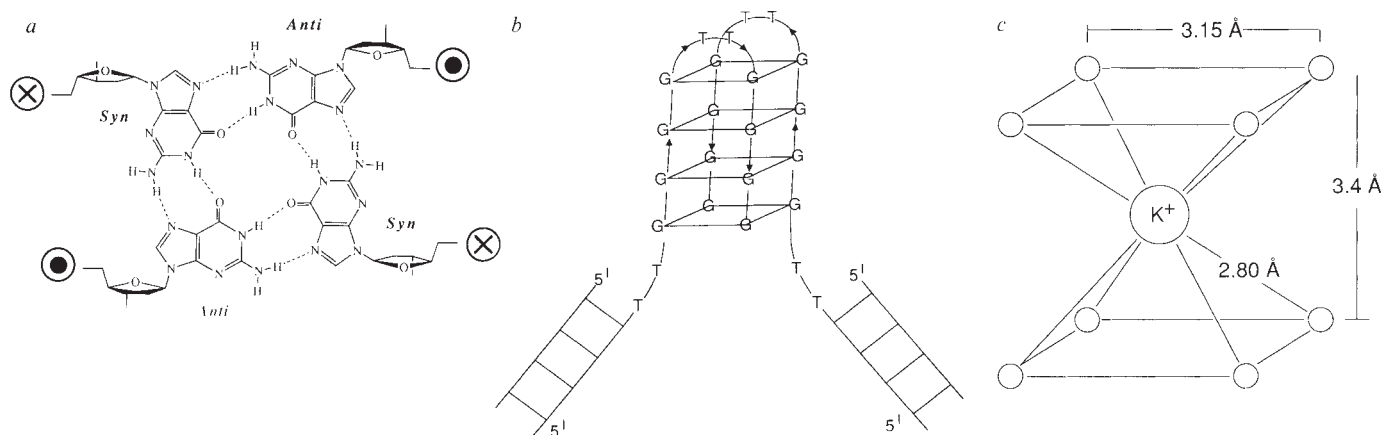


FIG. 4 *a*, Structure of a guanine tetrad within an antiparallel quadruplex. Relative strand polarities are represented 5' to 3' into (⊗) or out of (⊙) the plane of the paper. The diagram emphasizes the structural requirement that adjacent antiparallel strands must have different glycosidic conformations. The particular combination of strand polarities and glycosidic conformations shown in the diagram is arbitrary, however, and different choices give rise to structurally inequivalent quadruplexes. Antiparallel quadruplexes may also be constructed in which two adjacent strands of the same polarity and conformation pair with another two strands of the opposite polarity and conformation. Each theoretically possible overhang structure can pair with only a limited subset of the other possible structures and this may explain the slow rate of dimer formation. We note that adenine also has the potential to form an isostructural tetrad, albeit stabilized by only four (N6H...N1) hydrogen bonds, raising the possibility that adenines could also be incorpo-

ated into quadruplexes. *b*, Proposed structure of Tet1 hairpin dimers. The antiparallel quadruplex that mediates dimerization is shown with the cyclic guanine tetrads depicted schematically. The helical arms are shown projecting from the same side of the quadruplex as suggested by electrophoretic mobility, the hindered *Sma*I accessibility, and electron microscopic studies⁶. The arms are depicted in a *trans* configuration with respect to the guanine tetrads, but could alternatively be in a *cis* conformation. Note also that the quadruplex has two pairs of inequivalent grooves, either of which could, in principle, be spanned by the TT loops giving a further multiplicity of isomers. *c*, Eight-coordinate potassium chelation cage created by the O6 oxygen atoms of two stacked guanine tetrads. The interatomic distances were derived from fibre diffraction and model building studies of poly(G) (ref. 15). Note that the K-O bond length of 2.80 Å is independent of helical twist.

have the potential to form similar hairpin dimers. In most species, the guanine-rich telomeric strand terminates with simple repeats of the consensus sequence N_nG_m ($n \geq 2, m \geq 3$), although irregular repeats of similar sequences are found in several species^{1,2}. A 3' overhang of approximately two N_nG_m repeats is also conservatively maintained in species where the exact telomeric terminus is known or can be inferred^{3,4}.

Although the biological roles, if any, of antiparallel guanine quadruplexes remain uncertain, we envisage several possible instances where such structures may be used. First, there is considerable cytological evidence for the association of telomeres in both meiotic and mitotic cells¹. Our model offers a structural basis for the mediation of these associations by DNA-DNA recognition, although it seems reasonable to expect that telomere binding proteins may serve to repress, promote, or otherwise control these events *in vivo*^{19,20}. The present work also offers an explanation for the finding that exonuclease digestion of the cytosine-rich telomeric strand of *Oxytricha* inhibits subsequent telomere association⁷. Telomere dimerization could be inhibited if this digestion exposes four (or more) repeats on the guanine-rich strand, rather than the two normally present, because the strand could then fold back on itself to form an intramolecular antiparallel guanine quadruplex: that is, a covalently linked version of the hairpin dimer shown in Fig. 4*b*. Such structures, whether intra- or intermolecular, must now also be considered as potential substrates for telomerase^{21,22}, the enzyme that elongates telomeres²¹⁻²⁵.

The dimerization of two guanine-stabilized hairpin loops to form an antiparallel quadruplex could also provide a general mechanism for aligning and pairing two double helices within chromosomes in processes such as recombination. For two guanine-rich strands (one from each helix) to anneal into a quadruplex, however, their complementary strands must be displaced and stabilized, at least transiently, in a non-Watson-Crick structure. This stabilization could be effected by proteins that bind specifically to cytosine-rich, single-stranded DNA within recombinational enhancers²⁶, or by the formation of cytosine-rich hairpin loop structures, such as have been produced at low pH under conditions of superhelical stress²⁷. Other

unusual DNA structures are also known to create single-stranded stretches of guanines²⁸⁻³¹. The consensus sequence $G_mN_nG_m$ appears in a number of DNA sequences involved in recombination^{8,26,32-34}, suggesting that this sequence motif may also be used in some recombinational processes by forming guanine hairpin dimers as described here. □

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The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription

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TRANSCRIPTION of the *HO* gene of *Saccharomyces cerevisiae*, which encodes a site-specific endonuclease that initiates cell-type switching (reviewed in refs. 1,2), is restricted to a short window of the cell cycle in late G1 (refs 3,4). A repeated element in the upstream region of *HO* (the cell-cycle box, CCB) and two regulatory proteins, SWI4 and SWI6, are required for cell-cycle-dependent expression of *HO*^{5,6}. Biochemical experiments have identified a factor, CCBF (cell-cycle box factor), that binds to the CCB elements⁷ and that presumably plays a key part in cell-cycle regulation of *HO*. The *SWI4* and *SWI6* genes are required for formation of the CCBF-DNA complex. Here we report the nucleotide sequence of the *SWI4* gene and show that it contains two copies of the conserved SWI6-cdc10 motif observed in *SWI6* of budding yeast⁸, the *Schizosaccharomyces pombe cdc10* gene required for progression through G1⁹, the *Drosophila Notch* gene, and in the *Caenorhabditis elegans lin-12* and *glp-1* genes¹⁰⁻¹². We demonstrate by using antibodies to the SWI4 protein in gel-shift assays that the protein is present in the CCBF-DNA complex.

The nucleotide sequence and corresponding deduced amino-acid sequence of the *SWI4* gene are presented in Fig. 1. The single open reading frame of 3,282 base pairs encodes a potential polypeptide of 1,094 amino acids with a predicted relative molecular mass (M_r) of 123,730. The predicted SWI4 protein has a high overall asparagine content (14%). A region of 147 amino acids in the N-terminal one-third of the protein (indicated by small brackets in Fig. 1) has an overall Asn-Gln content of 42%. There is some evidence that polyglutamine stretches have a role in the activating capability of certain mammalian transcriptional regulators¹³. Although the precise role of the Asn-Gln stretch of SWI4 is not known, deletions of this region abolish the ability of the cloned gene to complement a *swi4*⁻ mutation (B.J.A., unpublished results).

The most interesting feature of the *SWI4* sequence was revealed by a search through available databases for proteins with sequences related to that of the *SWI4* gene product. The protein most similar to SWI4 is the product of the *cdc10* gene of *Schizosaccharomyces pombe*: SWI4 and *cdc10* are 26% identical over a 282-amino-acid region (Fig. 1a, b). The *SWI6* gene product shares a comparable degree of similarity to the *cdc10* polypeptide⁸. Neither SWI6 nor *cdc10* contain an Asn-Gln stretch comparable to that found in SWI4. The similarity between SWI4, SWI6 and *cdc10* is most significant (40-45% identity between SWI4 and *cdc10*) in two 33-amino-acid repeats (boxed in Fig. 1), the SWI6-cdc10 motif previously noted by Breeden and Nasmyth⁸ and observed also in the *Notch* and *lin-12* genes^{8,11}. SWI4 and SWI6 do not have extensive similarity outside the SWI6-cdc10 motifs. But, between these motifs, intermittent identities are found for SWI4, SWI6 and *cdc10* (see legend to Fig. 1b).

We have previously observed a correlation between SWI4 activity and CCBF activity; mutants defective in SWI4 lack CCBF activity and strains that carry the *SWI4* gene on a high-copy-number plasmid have increased CCBF activity⁷. We have now addressed the question of whether the SWI4 protein is present in this complex by using antibodies specific to SWI4.

These antibodies were prepared against a fusion protein containing the C-terminal 420 amino-acid residues of SWI4 fused to *E. coli* TrpE (Fig. 2). The antibody recognizes a protein in crude yeast extracts of about the size predicted for the SWI4 protein (M_r 120,000-130,000, on the basis of mobility). This protein is absent from extracts prepared from a strain carrying a nonsense mutation in the *SWI4* gene (*swi4-3*)⁷. A protein of reduced mobility was detected in strains bearing a truncated SWI4 derivative (*swi4*Δ143; Fig. 2, lane 3).

CCBF was identified using gel-retardation and footprinting experiments as an activity that binds to the CCB elements and that requires the *SWI4* and *SWI6* genes⁷. The gel-retardation experiment in Fig. 3 shows that the SWI4 protein is a component of the CCBF complex. Extracts from wild-type yeast were preincubated with preimmune or immune serum before inclusion in the CCBF-binding assay (described in ref. 7) and resolution of the complex on an acrylamide gel. If SWI4 is a component of the complex, the antibody might be expected either to interfere with complex formation or to impede the migration of the CCBF complex in the gel due to the presence of the antibody in the complex. Inclusion of SWI4 antibody in the CCBF-binding reaction resulted in the production of a complex of decreased mobility (Fig. 3, lanes 4-6, 8-9). No comparable alterations in band formation were seen in reactions containing extracts from *swi4*⁻ cells or extracts treated with preimmune serum (see Fig. 3). The new band in the lanes containing *swi4*⁻ extract corresponds to an N-terminal fragment of SWI4 that is produced in the *swi4*⁻ strain (see Fig. legend). These data demonstrate the presence of the SWI4 protein in the CCBF complex.

Of the six genes reported to contain the SWI6-cdc10 motif, the group from yeast (*SWI4*, *SWI6* and *cdc10*) is involved in regulation of events at the beginning of a new mitotic cell cycle, with the expression of *SWI4* and *SWI6* required for transcription of *HO*, and the expression of *cdc10* required for progression through G1. Our analysis provides information on the function of SWI4: it mediates cell cycle-dependent transcription as a component of a DNA-protein complex. It is plausible that *SWI6* and *cdc10* have similar functions. As noted by Breeden and

FIG. 1 DNA sequence and predicted protein product of the *SWI4* gene. **a**, Nucleotide sequences are shown extending 3,564 bases from a *HindIII* site upstream of the *SWI4* open reading frame to the termination codon. A region of high Asn-Gln content (14%) is enclosed by square brackets. Significant similarity between the SWI4 and *cdc10* polypeptides spans 282 amino acids (26% identity) and is enclosed in the dashed box. Two 33-amino-acid repeats within this region having 33-48% identity to corresponding sequences of both SWI6 and *cdc10* are enclosed in solid boxes. **b**, Alignment of the sequence (single-letter amino-acid code) of SWI4 (top line, sequence begins at amino-acid 472) and *cdc10* (bottom line, from amino-acid 311) in the region of clearly significant similarity (25.9% identity). Identical residues between the two sequences are boxed. Solid lines, the SWI6-cdc10 motifs. Amino acids that are also conserved in SWI6 in the first 50 residues after the N-terminal SWI6-cdc10 motif are shown by an asterisk above the residue. Residues conserved only between SWI4 and SWI6 in this region are also indicated (+). **c**, SWI4, SWI6 and *cdc10* are most similar within two 33-amino-acid motifs previously identified by Breeden and Nasmyth⁸ as a sequence motif occurring in the *SWI6*, *cdc10* and *Notch* gene products. Identical residues in all six repeats are boxed throughout all repeats and are in bold type. The identical residues in the most N-terminal repeats of all three proteins (repeat 1) are boxed as are the identical amino acids in the C-terminal repeats (repeat 2). The SWI4-1 repeat (amino-acids 514-546) is 45% identical to the SWI6-1 repeat and 48% identical to the *cdc10*-1 repeat. The SWI4-2 repeat (amino-acids 635-667) is 33% identical to the SWI6-2 repeat and 39% identical to the *cdc10*-2 repeat. **METHODS.** The *SWI4* gene was cloned by complementation of a *swi4* mutation as previously described⁷. Restriction fragments of the *SWI4* clone (B3.2, described in ref. 7) were subcloned into M13 vectors and sequenced by the dideoxy-chain-termination method using Sequenase (USB). The SWI4 protein sequence was aligned with proteins from the Dayhoff data base using the *dfastp* and *genalign* programs (Biomathematics Computation Lab, Computer Graphics Centre, UCSF).

a

1 AAG TTT TTT TTT TTT TCG AGA TAA TAA AAA AAG AAA CTC TTT AGT GTA TTG TGT TAT TTT
31
61 TAT TAT TAA TTG TTG TTA CAG CAG AAT CAC TCT AGC ATT GGT CGT CGG TTT TTC AAA CGA
91
121 TTC CTC CAC TTA CCG TTT GAA AAA AAG GTA CAT TAT TTC AGG TTA TTT GAT ATA TTG
151
181 TCA ACA ATA ATT GCT CTT TGC CGA AAA TTA ACT CTT CTT TCT TTC TCA TTT CCG TTC TGT
211
241 CCG TCT TGC GTA GTT CTA AAA GGT TGA TTT ATT CGA GAA ATC ATG CCA TTT GAT GTT TTG
271
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361 CTT TTG GCG CCT CAT TCA AAC CAT CCA HTG ATT GAA ATA GCT ACG TAT TCA GAA ACC GAT
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481 GAT TGG ATT AAT ATT ACC CAG GTT TTC AAA ATA GCT CAA TTT TCG AAG ACG AAA AGA ACT
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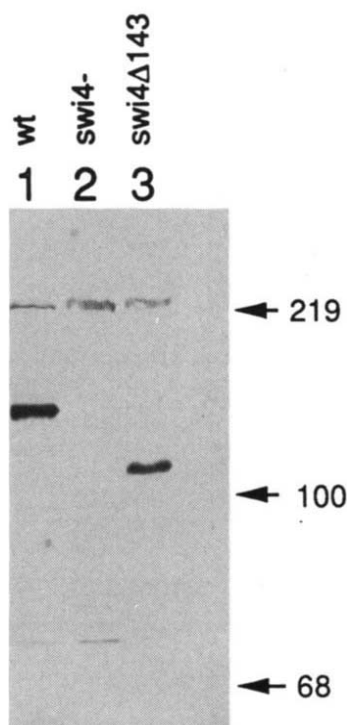


FIG. 2 Western blot of yeast extracts using SWI4 antibodies. lane 1, extract from a wild-type (wt) strain (strain 2000, α derivative of IH1998); lane 2, extract from a *swi4-3* strain (IH2008, which carries an amber mutation in *SWI4*, B.J.A., unpublished results); lane 3, extract from a *swi4-3* strain carrying a plasmid with a derivative of SWI4 truncated for 143 C-terminal amino acids. All lanes contain 100 μ g protein. Strains are as previously described^{7,16}. The approximate positions of migration of pre-stained *M_r* markers (BRL) are indicated (*M_r* $\times 10^{-3}$).

METHODS. Antibody was prepared using a fusion protein containing the C-terminal 420 residues of the SWI4 protein fused to *E. coli* TrpE expressed from plasmid pATH2 (gift of T. J. Koerner and A. Tzagaloff). The hybrid protein was induced with indoleacrylic acid and purified essentially as previously described¹⁷. The protein was isolated by gel electrophoresis, and eluted protein or protein embedded in gel was used to immunize rabbits (BabCo, Inc., Berkeley, California). For Western blots, yeast extracts were prepared as previously described⁷ and separated by SDS-PAGE. Proteins were transferred to nitrocellulose¹⁸. The filter was incubated with SWI4 antibody at 1:1000 dilution followed by goat anti-rabbit antibody conjugated to alkaline phosphatase (Boehringer). The C-terminal deletion of SWI4 was constructed by cutting plasmid B3.2 with *Eco*RI and religating. The resulting plasmid (p Δ Eco) is deleted for nucleotides corresponding to the 143 C-terminal residues of SWI4.

Nasmyth⁸, *SWI4* and *SWI6* are functionally redundant in two respects. First, the cloned *SWI4* gene can complement a *swi6*⁻ defect. Furthermore, even though strains with null mutations in either *SWI4* or *SWI6* are viable, the *swi4*⁻ *swi6*⁻ double mutant is inviable^{6,7,14}. Because *SWI4* and *SWI6* share sequence similarity and are functionally overlapping, it seems quite possible that SWI6 will also turn out to be part of the CCBF. Although *SWI4* and *SWI6* are functionally overlapping, they are not fully redundant, because expression of *SWI6* on a high-copy-number plasmid cannot compensate for a *swi4*⁻ defect (B.J.A., unpublished results). Breeden and Nasmyth⁸ suggested that the overlapping and essential function carried out by SWI4 and SWI6 (inferred from the inviability of *swi4*⁻ *swi6*⁻ strains) might be the same essential function as carried out in fission yeast by *cdc10*. More specifically, SWI4 and SWI6 may be required for transcription of genes such as DNA polymerase I (which are expressed at the same time in the cell cycle as *HO*)¹⁵ and *cdc10* may have a similar role.

In contrast to *SWI4*, *SWI6* and *cdc10*, the other group of

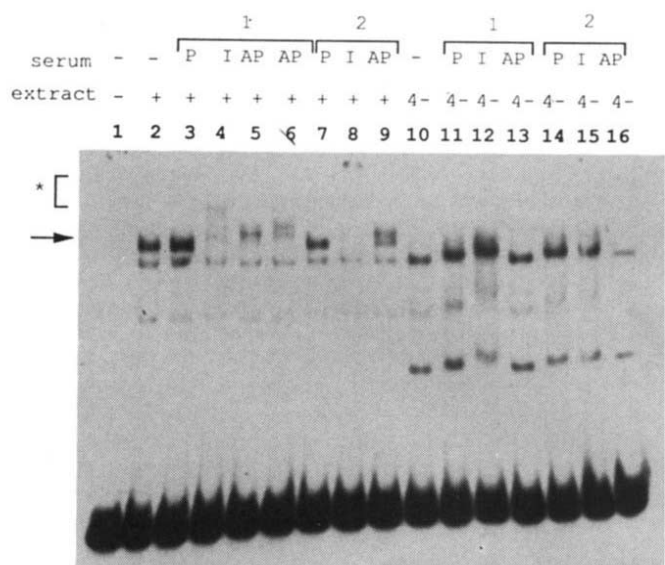


FIG. 3 SWI4 is a component of CCBF. Gel electrophoresis of complexes formed with a probe containing three CCB elements and surrounding *HO* upstream regulatory sequences (120-base-pair probe from plasmid pBA128, ref. 7) and extracts from either wild-type (wt) or *swi4*⁻ (4-) yeast. The same wild-type extract was used in the experiments shown in Figs 2 and 3. The *swi4*⁻ extract was made from strain BY171 (α *HO::lacZ swi4-1 trp1 leu2-ura3-52 ade5*) which carries a nonsense mutation (*swi4-1*) in *SWI4*. This mutation results in the production of an N-terminal fragment of SWI4 that is nonfunctional *in vivo*, does not support CCBF complex formation *in vitro* (lanes 10, 14), but can still bind to the CCB sequences to create a complex of decreased mobility. The SWI4 antiserum was raised to the C-terminus of SWI4 and does not recognize this truncated species (lanes 11, 12, 15 and 16, and data not shown). Binding reactions were preincubated with preimmune serum (P), immune serum (I), affinity purified antiserum (AP) or buffer (-) as indicated. The CCBF complex is indicated by the arrow and the antibody-shift derivatives of CCBF by an asterisk-marked bracket. Free probe is the lowermost band on the gel.

METHODS. All binding reactions were performed as previously described⁷ and contained 10 μ g yeast extract. Antibody preincubations were performed essentially as described by Bender and Sprague¹⁹. Extracts were incubated with nonspecific competitor for 5 min on ice before the addition of 0.05 μ l preimmune serum (lanes 3, 7, 11, 14), 0.1 μ l immune serum (lanes 4, 8, 12, 15) or 0.1 μ l affinity-purified antiserum (lanes 5, 9, 13, 16). Lane 6 contains 0.4 μ l dilute preparation of affinity-purified antiserum. The sera were prepared from two different rabbits (designated by brackets and the numbers 1 and 2 above the lanes). After 10 min at room temperature, radioactive probe was added, and the reactions were incubated for an additional 10 min before gel electrophoresis⁷. SWI4 antiserum was affinity purified essentially as described²⁰, except that antibody was eluted from nitrocellulose strips with bound SWI4::TrpE by incubation in 4 mM MgCl₂, 50 μ g ml⁻¹ BSA for 30 min at room temperature. Affinity-purified antibody was dialysed against 10 mM Tris-HCl, pH 7.4, 150 mM NaCl and concentrated in a Centricon ultrafiltration unit (Amicon).

genes (*Notch*, *lin-12* and *glp-1*) is involved in decisions that take place during development and codes for proteins that could be receptors associated with the cell membrane. The SWI6-cdc10 motif is present in these proteins in six tandem copies. Unless the segment containing the SWI6-cdc10 motif is released from its membrane anchor, this motif must function in at least two different cellular locations for the six proteins so far described. This motif could have been conserved because it interacts with another conserved protein. In the same way as upstream regulatory regions seem to have evolved as collections of short segments conferring different modes of regulation, proteins could also be composites made up of short functional elements. The SWI6-cdc10 motif could be one such element that, for example, monitors position in the cell cycle by being a target for a protein kinase. Further studies of CCBF should allow identification of such an interacting component. □

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A novel group of abyssal methanogenic archaeobacteria (*Methanopyrus*) growing at 110 °C

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THE organisms with the highest growth temperature known so far are members of the archaeobacterial genus *Pyrodicticum*^{1,2}. These anaerobic sulphur reducers thrive at temperatures of up to 110 °C within a shallow hydrothermal system off Vulcano, Italy. Only a few hyperthermophilic methanogens are known—members of the genus *Methanothermobacter*, which grow exclusively within terrestrial fields of fumaroles from which sulphurous gas is emitted and show an upper growth temperature of 97 °C (ref. 3), and some members of the genus *Methanococcus*, which grow within deep-sea hydrothermal systems at temperatures up to about 90 °C (ref. 4). We have now isolated a novel group of methanogenic archaeobacteria growing at least at 110 °C from sediment samples taken by the research submersible *Alvin* at the Guaymas Basin hot vents (Gulf of California). This finding demonstrates the unexpected biogenic methanogenesis at temperatures above 100 °C, and, in view of biogeochemistry, could explain isotope discrimination at temperatures that were thought to be unfavourable for biological methanogenesis.

During studies of the microbial ecosystems within hydrothermal areas, sediment core samples were taken by the research submersible *Alvin* (dive nos. 1,966–1,971) at the Guaymas hot vents (27°00' N, 111°24' W; depth, 2,000–2,010 m). The *in situ* temperatures of the sampled sediments were between 7 °C and 140 °C depending on the sites and depths of coring. The cores

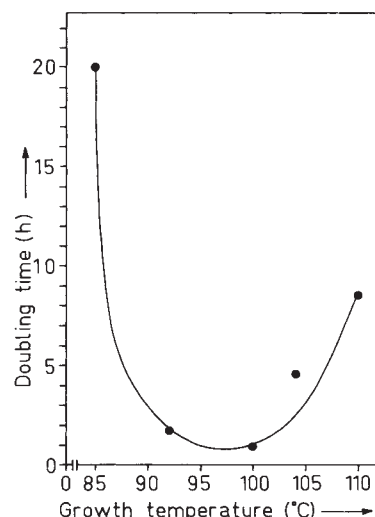
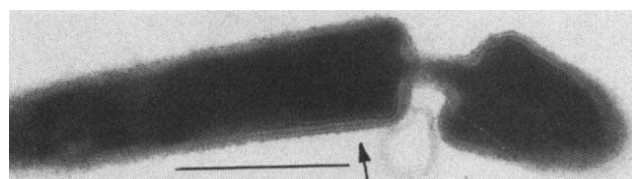


FIG. 2 Temperature dependence of growth of isolate AV 19. The doubling times were calculated from the slopes of the exponential growth curves (not shown). Cells were grown anaerobically⁸ in 120 ml stoppered serum bottles containing 10 ml mineral medium³ supplemented with 1.5% NaCl. The cultures were shaken (200 r.p.m.) with H₂-CO₂ (80:20 v/v; 300 kPa) as a gas phase. Temperatures were observed with a gauged thermometer (Brand, Wertheim). Growth was followed by cell counting in a 'Thoma' chamber (depth, 0.02 mm).

were processed in the laboratory of the research vessel *Atlantis II*. They appeared blackish and smelled of sulphide and crude oil. The thermogenic origin of petroleum-type hydrocarbons in these sediments have been reported earlier^{5,6}. Subsamples from various core sections were immediately transferred into 100 ml storage bottles, which were then sealed with rubber stoppers. Traces of oxygen were removed by injecting sodium dithionite⁷.

In the home laboratory, anaerobic sterile mineral medium³ supplemented with 1.5% NaCl was inoculated with the samples (5% inoculation) and incubated at 100 °C while being shaken (200 r.p.m.) in the presence of an H₂-CO₂ atmosphere (80:20 v/v; 300 kPa). After 1–3 days, whitish flocs became visible within the culture attempts of five samples, which had original temperatures of between 85 °C and 140 °C. Microscopic inspection revealed the presence of short chains of rod-shaped bacteria (termed isolates AV 19), often sticking together in raft-like aggregates. The individual cells were ~8–10 µm long and 0.5 µm in width. Under the ultraviolet microscope at 420 nm the bacteria showed a bluish green fluorescence typical of methanogens⁸. The enrichment cultures were purified by serial dilution in the same medium. The cultures formed ~86 µmol methane per ml, indicating that the organisms present were methanogens. The cells were motile and were positive for Gram-staining. Ultra-thin sections of the cells under the electron microscope revealed an unusual double-layered cell envelope that covered the membrane (Fig. 1). The novel methanogens grew between 84 °C and 110 °C with an optimum growth temperature of ~98 °C (50-min doubling time). At 110 °C there was still vigorous growth with a doubling time of only 8 h (Fig. 2). No growth was observed at 80 °C and at 115 °C. The organisms grew in the presence of 0.2–5% NaCl (optimum, ~1.5%; Fig. 3) and

FIG. 1 Electron micrograph of a thin section of isolate AV 19. The sections were doubly contrasted with lead citrate and uranyl acetate. An unusual double-layered cell envelope covers the membrane (arrow). Scale bar, 1 µm.



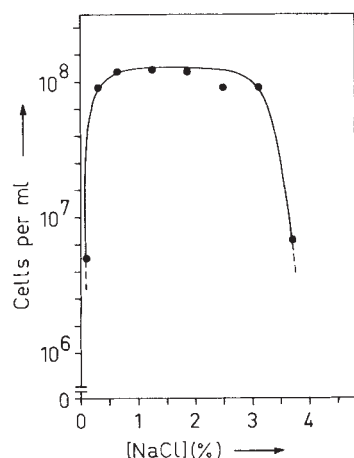


FIG. 3 Influence of salt on growth of isolate AV 19. Cultures were grown at 100 °C as described in Fig. 2 but at different salt concentrations.

at pH 5.5–8 (optimum, pH 6.5). Growth of the new isolates was strongly inhibited by traces of oxygen. They were only able to use H_2 and CO_2 as energy and carbon sources and were therefore chemolithoautotrophs. The cell membrane contained one predominant lipid, which was identified as 2, 3-biphytanyl glycerol (A. Gambacorta, personal communication). The G+C content of the DNA was 60 mol %. Further chemical analysis demonstrated the presence of 1.1 M cyclic 2, 3-diphosphoglycerate (DPG) within the cells of the new isolates. Previously, only much lower amounts (0.05–0.3 M) of DPG had been found within mesophilic and thermophilic methanogens, and were thought to be responsible at least in part for the thermostability of enzymes^{9,10}. The presence of tremendous amounts of DPG in the novel hyperthermophilic methanogens reported here could support this assumption. The phylogenetic studies by comparison of 16S ribosomal RNA sequences indicate that the new isolates show no closer relationship to any methanogen known so far (C. R. Woese, personal communication), and the new genus *Methanopyrus* is proposed for them. The novel methanogens are found at the particular temperatures and pressures of the Guaymas Basin hydrothermal vent site where terrestrial as well as planktonic sediments accumulate at high rates¹¹. Pyrolysis of organic materials, especially diatomaceous lipids, is believed to lead to the production of the petroleum-type hydrocarbons that are characteristic of these sediments^{5,6}. Under these conditions the new isolates could be responsible for methane formation at temperatures between 90 °C and 110 °C. Because of their broad salt tolerance, the new isolates could be generally involved in the formation of methane in marine as well as in freshwater hydrothermal environments accompanying oil production under elevated temperatures and pressures. □

Massive natural occurrence of unusually large bacteria (*Beggiatoa* sp.) at a hydrothermal deep-sea vent site

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WHITE web-like 'mats' of the filamentous sulphur-oxidizing bacterium *Beggiatoa* are commonly observed on the surface of anoxic sediments^{1–3}. As a typical interface organism *Beggiatoa* requires a source of inorganic reduced sulphur and dissolved free oxygen. The first pure cultures of marine strains of *Beggiatoa* were recently obtained by artificially reconstructing an O_2 – H_2S interface in a semi-solid medium that supports the gliding mobility of the filaments^{4,5}. The maximal thickness of these *Beggiatoa* mats in culture was 1.0 mm. We now report the discovery of dense layers of filamentous sulphur-oxidizing bacteria up to 3 cm thick on the sediment surface, and up to 30 cm thick between stands of vestimentiferan tube worms at the Guaymas Basin hydrothermal vent site in the Gulf of California at a depth of 2,010 m. The mats are essentially monocultures of *Beggiatoa*-type organisms containing filaments of three width classes, the largest filaments being 116–122 µm in diameter. Freshly collected filaments showed chemoautotrophic metabolism and active gliding motility. The phenomenon of a natural mass growth of a bacterium is of great physiological and ecological interest, and could also be of biotechnical importance considering the difficulties of mass cultivation of interface organisms such as *Beggiatoa* and the other 'large' sulphur-oxidizing bacteria such as *Thiovulum*⁶ and *Thioploca*⁷.

The occurrence of *Beggiatoa* in quantities several orders of magnitude higher than those previously observed in nature or achieved in the laboratory is not only highly unusual but could also provide physiological and technical information. In fact, there is no other example of a virtual bacterial monoculture growing under natural conditions at such a pure state (that is, not mixed with sediment and living or dead cells of other mat-forming organisms as in stromatolites) that it can be harvested, literally, by vacuuming. Although white and yellow microbial mats were first observed at the Guaymas Basin in 1983 (refs 8, 9), only recently has there been information on the identity of the organisms and their metabolic characteristics. This information resulted from shipboard studies on material

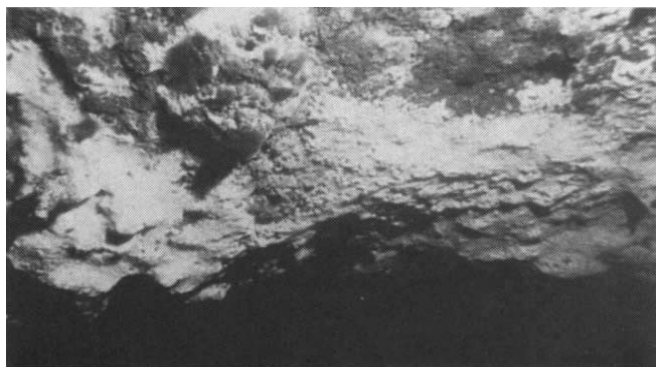


FIG. 1 *Beggiatoa* mats covering hydrothermal sediments at the Guaymas Basin vent site (depth, 2,006 m).

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sampled during a series of dives with the research submersible *Alvin* in 1985 and 1988 (ref. 10).

This unusual mass occurrence of *Beggiatoa* could be due to (1) the more efficient mass transfer of H_2S and O_2 in a flow system than in a diffusive system, (2) a continuous supply of an organic growth factor, (3) facultative chemoautotrophy providing an efficient metabolic flexibility in the case of an intermittent or turbulent vent flow, or (4) an optimal temperature gradient.

It is characteristic of the Guaymas Basin site that the hydrothermal fluid has to pass through a 300–400-m layer of sediment before venting at the sea floor either in the form of hot 'smokers' of up to 350 °C, or slow seepages¹¹. The latter are highly variable. Shimmering heat 'schlieren' can indicate water extrusion of 5–20 °C above ambient temperature (2 °C) with sediment temperatures of ~200 °C at a depth of 40 cm. At sites of slower seepages the temperature at the sediment surface may not be different from ambient temperature and can reach 40–50 °C at depths of 40 cm. The chemical composition of the Guaymas Basin vent waters is strongly affected by its passage through the organic-rich terrestrial and planktonic (diatomaceous) sediments (Table 1). The en route deposition of polymetal sulphides lowers the H_2S as well as iron and manganese concentration as compared with those of the 'bare lava' vent emissions, turning the 'black smokers' transparent. Microbiologically, even more important is the presence of ammonia as an additional source of nitrogen. Also characteristic for the hydrothermal sediment of the Guaymas Basin is the spotty occurrence of thermogenic petroleum that seems to result in the presence of an active hydrocarbon-degrading microbial population in the top sediment layer^{12,13}.

The patches of *Beggiatoa* mats covering the anoxic black and, in spots, warm sediment surface reach 3–4 m in diameter (Fig. 1). They appear brightly yellow or orange in the spotlight of *Alvin* and were found to contain high concentrations of c-type cytochromes¹⁴. Layers of bacterial filaments up to 30-cm thick engulf stands of tube worms (*Riftia* sp.), often covering them entirely except at the retractable gill section. When drifting off in the currents caused by the submersible, these assemblies of bacterial filaments resembled flocks of glass fibres used for insulation (Fig. 2). The 120- μ m diameter filaments are readily visible in the reflecting lights of the submersible. We collected these cell suspensions in 12-l quantities using a 'slurp gun' (a 7-cm diameter flexible corrugated hose attached to a glass jar in the basket of *Alvin* fitted with a suction pump and a 1-mm mesh plankton net covering the exhaust). The hose intake was

TABLE 1 Concentrations of chemical species in hydrothermal vent emissions from sediment-covered Guaymas Basin (GB) and 'bare-lava' 21° N vent site of the East Pacific Rise spreading zone compared with those of ambient seawater (SW)

Chemical	Concentrations*		
	GB (2,003 m)	21° N (2,610 m)	SW
Fe	56	1,664	0.001
Mn	139	960	0.001
Mg	29	0	52.7
SO_4^{2-}	0	0	27.9
NH_3	15.6	0	0
H_2S	5.82	7.3	0
pH	5.9	3.4	8.0

Data from ref. 11.

* Concentrations of Fe and Mn in μ M; those of Mg, SO_4^{2-} , NH_3 and H_2S are in mM.

guided by the mechanical arm of *Alvin*. The jar was filled with a slightly viscous cell mass in a few minutes.

After a retrieval to the surface lasting ~1.5 h, the dense cell suspension was microscopically and physiologically studied in the ship's laboratory¹⁰. The *Beggiatoa* filaments occurred in three main width classes, at least two of which were potentially chemoautotrophic as demonstrated by $^{14}CO_2$ fixation measurements, autoradiography and the presence of Calvin-cycle enzymes. The activity of ribulose 1, 5-bisphosphate carboxylase, based on cell protein, measured in these natural filament assemblies was ~two-thirds of that measured in obligate chemoautotrophic bacteria^{10,15}. The facultative heterotrophy of *Beggiatoa*⁴ and the abundance of dissolved organic substrates in the top sediments of the Guaymas Basin as well as in the immediate surrounding of the tube worms could play a critical part in the mass growth of these organisms.

The unusually large diameter of some of the cell filaments can be interpreted as an adaptation to this extreme environment. The amount of cytoplasm in these cells is relatively small, and all of it is distributed along the outer cell wall (Fig. 3) for all three width classes. For the cells shown in Fig. 3, 16.7% (small cell) and 12.5% (large cell) of the cross-sectional area is composed of cytoplasm and cell wall, and this is representative of the entire population as judged by the ratio of protein content to filament volume¹⁰. The inner space of the cells seems to be filled by a large liquid vacuole. This could allow the cells to grow to an enormous size without diffusional problems, thus

FIG. 2 Virtual monoculture of *Beggiatoa* sp. filling the space between the vestimentiferan tube worms (*Riftia* sp.) with tube lengths here of ~30–40 cm. Flocks of bacterial filaments (top center and right) are dislodged by the research submersible (depth, 2,010 m).



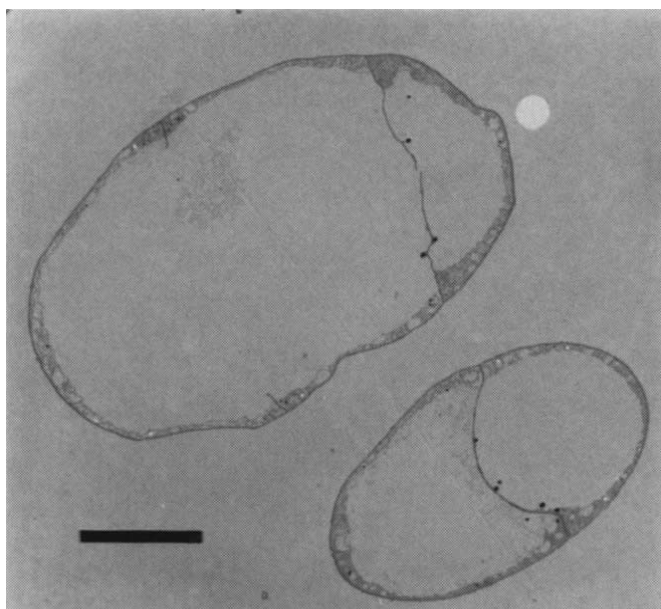


FIG. 3 Transmission micrograph of *Beggiatoa* filaments from the Guaymas Basin vent site showing the enormous liquid vacuole (scale bar, 10 μ m).

giving the organism the structural rigidity necessary to exploit a larger ambient space than can smaller filaments without support of a substratum (for example, sediment). Moreover, an inner low-redox reservoir could make it possible for the organisms to sustain the absence of reduced chemical species during flushing by oxygenated ambient seawater for considerable periods of time. Experiments to address these questions require the obtaining of pure cultures, which has not so far been achieved.

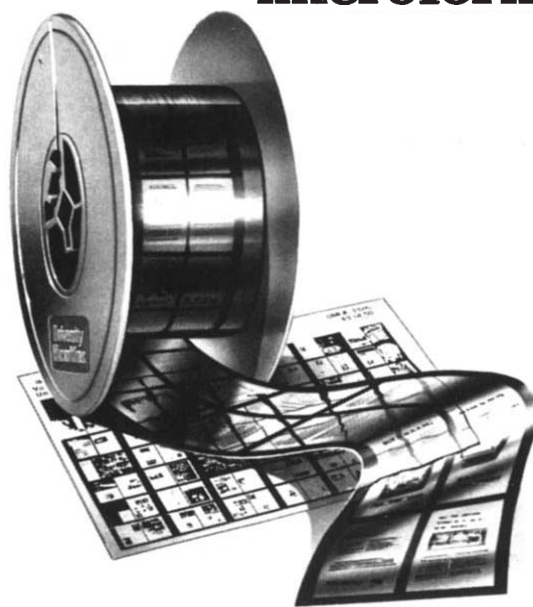
Continued studies of this natural mass occurrence of *Beggiatoa* could lead to an understanding of the mode of biological sulphur oxidation, its strategy in competing with the spontaneous chemical oxidation, and the role of possible organic growth requirements of the organisms involved. The ecological interest of this phenomenon concerns the role of the chemoautotrophic production of organic carbon by *Beggiatoa* and its transfer to grazing animals at the sediment-covered Guaymas Basin deep-sea vents. This is in contrast to those chemosynthetic processes that are predominant at the offshore bare-lava hydrothermal vent sites⁸ where *Beggiatoa* has been rarely observed. □

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The GUS reporter gene system

R. A. Jefferson

The GUS reporter gene system is already a powerful tool for the assessment of gene activity in transgenic plants. Further developments may lead to routine *in vivo* analysis and fusion genetics.

THE GUS (β -glucuronidase) gene fusion system is now being used extensively as a reporter gene in plant and agricultural molecular biology. Here, I review the properties of GUS that have led to its widespread use and discuss possible developments of the system.

The enzyme β -glucuronidase (GUS E.C.3.2.1.31) catalyses the hydrolysis of a wide variety of glucuronides. Its substrates consist of D-glucuronic acid conjugated through a β -O-glycosidic linkage to virtually any aglycone. Most naturally occurring substrates for GUS are generated by the main detoxification pathway of vertebrates; thousands of endogenous and xenobiotic compounds, including steroid hormones, bilirubin, plant secondary products and antibiotics, are conjugated to glucuronic acid in the liver and other organs of vertebrates to effect detoxification, and are then excreted in the urine and bile^{1,2}.

Escherichia coli, one of the most abundant aerobic constituents of the vertebrate large intestine, is unusual among bacteria in possessing a highly efficient mechanism for the assimilation and metabolism of the many β -glucuronides with which it is presented. The gene encoding β -glucuronidase from *E. coli* K12, *gusA* (formerly *uidA*) has been sequenced and the gene product shown to be a stable homotetramer of subunit relative molecular mass of 68,000³.

Advantages of GUS over other reporter systems include the robustness of the enzyme, the simplicity of the assays and the variety of substrates available. These include sensitive histochemical substrates such as 5-bromo-4-chloro-3-indolyl β -D-glucuronide (X-Gluc; Fig. 1), and numerous other chromogenic and fluorogenic substrates for quantitative analysis. Expression of the gene is not deleterious to plant hosts⁴. In addition, virtually all aspects of the GUS gene fusion system can be reduced to very low technology as many GUS substrates can be prepared biosynthetically using the conjugation and excretion systems of vertebrates.

A key advantage of GUS is the absence of GUS activity in many organisms other than vertebrates and their attendant microflora. Lower and higher plants and most bacteria, fungi and many insects that exist in the phyllo- and rhizosphere are largely, if not completely lacking in GUS activity⁴. Minute quantities of GUS activity can therefore be accurately

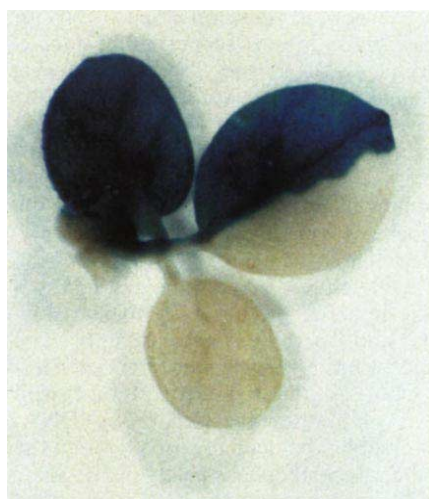


FIG. 1 GUS expression in leaves of a R₁ soybean plant transformed with a CaMV 35S promoter — GUS fusion. The leaf was incubated with the substrate X-Gluc, which is cleaved in GUS-expressing cells resulting in deposition of a blue indigo dye. The pattern of GUS expression indicates that this plant is a chimaera, and this has been confirmed by DNA hybridization. The lack of background activity in non-transformed cells is clear. (Courtesy of P. Christou and W. Swain Agracetus.)

measured — even in single cells⁵ — when *gusA* is used as a reporter gene in these systems.

Applications

Since the development of the GUS system, many thousands of transgenic plants have been generated expressing GUS. In such plants, the spatial distribution of gene activity can be visualized in the absence of any background signal. This has been instrumental in the development of new methods of plant transformation such as particle bombardment⁶⁻⁸, and in the successful transformation of many crop plants including soybean and monocots such as rice⁸⁻¹⁰. The power and simplicity of GUS histochemical methods is also demonstrated in assays for transposon excision¹¹, lineage analysis^{6,12} (Fig. 1), developmental analyses¹³ and discrimination between gene family members¹⁴.

GUS fusions are now widely used to study plant-pathogen and plant-symbiont interactions. They can be used both to study expression of particular genes and to mark and monitor populations of micro-organisms in soil or in association with plants. GUS offers a promising route for transformation, monitoring and molecular

genetic analysis of the numerous important epi- and endophytic organisms that are not readily cultured, such as oomycetes and mycorrhizal fungi. In addition GUS fusions can be used in many laboratory model systems lacking endogenous activity, including *Saccharomyces*, *Schizosaccharomyces*, *Dictyostelium*, some strains of *Caenorhabditis elegans*¹⁵ and *Drosophila*.

Unlike *lac Z*-encoded β -galactosidase, *gus A*-encoded GUS can readily traverse membranes when an appropriate transit or signal sequence is fused to its amino terminus. GUS fusions have been used to study targeting and transport of chimaeric proteins into chloroplasts¹⁶, mitochondria¹⁷ and the endoplasmic reticulum (ER)¹⁸. The N-terminal addition of an ER targeting sequence may lead to the secretion of GUS from cells: however, in the ER, GUS becomes glycosylated at two cryptic N-linked glycosylation sites and is thereby inactivated¹⁸. Work is in progress to eliminate these cryptic sites to allow fully active GUS to be secreted. Secreted GUS may then accumulate in the space between the plant cell membrane and cell wall, where it can act upon those soluble substrates that can cross the cell wall.

Glucuronide permease

E. coli has evolved not only the *gusA*-encoded hydrolase, but a general mechanism for transmembrane transport of β -glucuronides. The glucuronide permease was first described biochemically by Stoeber¹⁹, and has recently been characterized genetically and molecularly (R.A.J. unpublished). It can accumulate a wide variety of β -glucuronide substrates ranging from simple aliphatic to large, complex heterocyclic conjugates. The 49 kDa permease is encoded by a single gene, *gusB*, which is cocistronic with *gusA* (R.A.J. and W. Liang, in preparation), and is an integral membrane protein that appears to be a symporter coupled to the H^+/Na^+ gradient.

It seems likely that glucuronide permease will also function in heterologous systems, including plants. This would permit the accumulation of GUS substrates in cells, and would enable quantitative, non-destructive, real-time imaging of gene activity within the organism. New fluorogenic substrates for GUS, such as trifluoromethylumbelliferyl glucuronide and resorufin glucuronide (Molecular Probes, Inc.) that are efficiently trans-

ported by the permease and fluoresce maximally at the pH of the cell, together with recent developments in epifluorescence microscopy and imaging, should be of value in this respect.

Fusion genetics

Application of genetic selection to obtain *cis*- and *trans*-acting mutations that affect gene fusion activity in genetically complex eukaryotes has been described by Bonner *et al.*²⁰ in *Drosophila*. The potential use of GUS for this approach hinges on the ability to prepare hundreds of different substrates that could release bioactive aglycones on hydrolysis by GUS, so giving the possibility of implementing almost any conceivable selection.

For example, plant growth regulator glucuronide conjugates have been synthesized and shown to provide hormonal effects only to those tissues expressing GUS (R. A.J., unpublished). Conversely, cytotoxic aglycones such as cycloheximide or a herbicide could be conjugated to give a non-toxic but toxicogenic suicide substrate that kills GUS-expressing cells specifically. One can envisage targeting GUS activity to discrete cells, tissues, plants or developmental stages, thereby targeting the effects of a generally applied substrate. This would provide not only genetic selections but may allow new means of modifying the physiology and/or agronomic traits of a plant. □

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DNA technology transfer

This week's focus on recombinant DNA technology features a protocol for the rapid separation of plasmid DNA, a baculovirus expression system and customized RNA-oligos.

HYDROLINK is the new alternative to agarose and acrylamide gels, introduced by Biochem and developed specifically for the electrophoretic separation of double-stranded DNA and DNA sequencing (Reader Service No. 101). HL-dsDNA is a novel polymer supplied as a ready-to-pour stable liquid, which can be used to separate dsDNA from 100–7,000 bp, with nonlinear separation up to 23,000 bp. Biochem says that compared with polyacrylamide and agarose gels, HydroLink offers a ten-fold increase in loading capacity, higher resolution, picomole sensitivity, and increased gel strength. The HydroLink DNA Sequencing Gel Electrophoresis System (HL-SEQ) comes with high pH buffer for denaturing DNA, eliminating the need for urea. Due to the chemical and thermal stability of HL-SEQ, sequencing parameters can be extended with respect to solvent, ionic strength, pH denaturing additives and temperature.

Using the new protocol for plasmid separation, plasmid DNA can be separated in 1.5 hours, says Jouan (Reader Service No. 102). Jouan, distributors of Hitachi's CS120 ultracentrifuge, has developed a method using CsCl gradients that cuts down on processing time and allows several runs per day. High-density CsCl is loaded into a tube and low-density CsCl is loaded on top. The plasmid DNA mixture is added and the tubes are spun in the CS120 at 120,000 r.p.m., 600,000 g for 1.5 hours. The two CsCl solutions form density gradients, but the boundary between the two solutions presents a significant step in densities, which, Jouan says, ensures good separation between the sharply defined bands of linear DNA (upper band) and circular DNA (lower band), which would normally be difficult to separate.

Using the UVC 1000 UV Crosslinker, DNA and RNA can be crosslinked to membranes in seconds not hours, says Hoefer Scientific Instruments (Reader Service No. 103). The UVC 1000 can accommodate a variety of membrane sizes, including blots from DNA sequencing gels. It can be used to crosslink to nitrocellulose, nylon, or nylon-reinforced nitrocellulose membranes for multiple probing of Southern, Northern and dot/slot blots. The machine's monitoring device can be preset to measure either energy in microjoules or time in minutes, with values displayed on the LED countdown display monitor. If set to measure energy, the UV



UVC 1000: crosslinks DNA and RNA to membranes in moments.

light is automatically deactivated when the energy level has been reached. The \$1,295 (US) UVC 1000, measuring 37 × 15 × 49 cm, is fitted with a door that is safety interlocked to prevent accidental exposure to UV.

Recombinant reagents

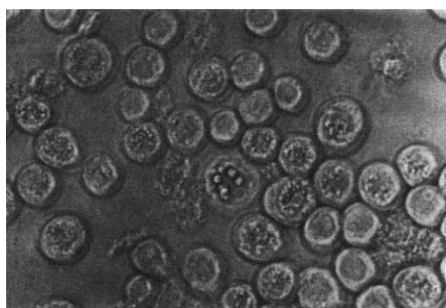
For researchers studying the mechanisms controlling cellular proliferation, Immunodiagnostic Systems Ltd is selling recombinant transforming growth factor (TGF-Beta 1) (Reader Service No. 104). TGF-Beta 1 is prepared from CHO cells using recombinant DNA techniques. It is an acid-stable, disulphide-linked 25 kDa dimer that is made up of two identical 12.5 kDa subunits, each comprising 112 amino acids. IDS says the recombinant TGF-Beta 1 has a purity of greater than 95 per cent when assayed by an amino acid sequencer and SDS-PAGE, and is free of the biohazards associated with natural TGF-Betas. Recombinant TGF-Beta 1 will be available in 1- and 5-µg quantities in early 1990.

The Japanese company Nacalai Tesque, Inc. is offering recombinant human prolactin (hPRL) (Reader Service No. 105). This simple polypeptide of 200 amino acids and molecular weight of 23 kDa is produced in *Escherichia coli* cells. As the hPRL is produced by recombinant techniques, it has, says Nacalai, a purity of greater than 99 per cent on SDS-PAGE and is free of contaminating protein, such as growth hormone, which may contaminate hPRL derived from the anterior pituitary gland. The recombinant hPRL is supplied in 100-µg, 1-, 10- and 100-g quantities as a lyophilized reagent that is stable at –20 °C.

In kit form

Invitrogen has introduced the MaxBac baculovirus expression system for the pro-

duction of proteins from cloned genes (*Reader Service No. 106*). The system utilizes the *Autographa californica* nuclear polyhedrosis virus (AcNPV) regulated polyhedrin promoter to 'drive' the expression of foreign genes. The recombinant



Invitrogen's Max Bax baculovirus expression system comes in kit form.

transfer vector containing the polyhedral gene sequence and the gene of interest is co-transfected with the wild-type AcPHV DNA. Homologous recombination results in the expression of the desired genes. Recombinant baculovirus can be screened either visually or immunologically. Protein expression and modification occurs after infection of *Spodoptera frugiperda* cells. The MaxBax system offers, says Invitrogen, several advantages over bacterial, yeast and mammalian systems: prokaryotic or eukaryotic sequences expressed as fused or non-fused recombinant proteins are immunologically and functionally similar to their authentic counterparts, baculoviruses are non-pathogenic to vertebrates or plants, and the system utilizes many of protein modification, processing and transport systems present in higher eukaryotes. The \$625 (US) MaxBac system comes complete with all reagents, manual and licence for research.

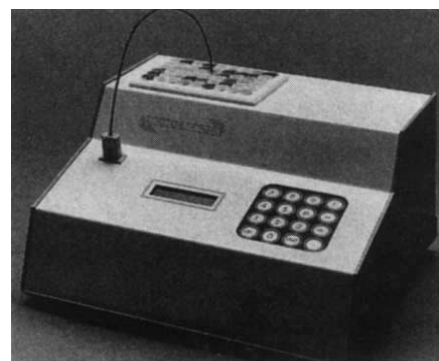
The T7-GEN *In Vitro* Mutagenesis Kit for **oligonucleotide-directed, site-specific mutagenesis** is now available from USB (*Reader Service No. 107*). The protocol, which takes less than four hours, involves annealing the mutant oligonucleotide primer to the single-stranded template. The primer is extended with T7 DNA polymerase in the presence of 5-methyl-dCTP, deoxynucleoside triphosphates and T4 DNA ligase, producing a double-stranded heteroduplex. The non-mutant parental strand is nicked with *Msp* I and removed by digestion with exonuclease III. Partially copied and contaminating single-stranded molecules are removed with *Hpa* I. Methylated single-stranded mutant DNA is transformed into a non-restricting strain of *E. coli*. USB says, the kit produces mutation frequencies of greater than 75 per cent. Mutations can be identified directly by nucleotide sequencing. The \$315 (US) kit is supplied with enough reagents for 20 mutation reactions and includes a control primer and tem-

plate for monitoring the test performance.

DNA amplification

Bios Corp. has introduced **PCRable DNAs** derived from a panel of somatic cell hybrids for use in *in vitro* amplification experiments (*Reader Service No. 108*). The PCRable DNAs allow the chromosomal localization of probes complementary to human target sequences present in hybrid cells, eliminating, says Bios, the delays in obtaining cell lines. Each \$1,500 (US) set comprises test protocol, 10 µg each of human and hamster control DNAs for the optimization of test conditions, and 5 µg each of 25 somatic cell hybrid cell lines — enough to perform 50–100 localizations.

The Hybaid Thermal Reactor is the new microprocessor-controlled **heating and cooling block** from Hybaid Ltd (*Reader Service No. 109*). The instrument, costing £2,495 (UK), has a maximum heating rate of 2.5 °C per second and a block uniformity of ±0.5 °C, says Hybaid. Temperature



Hybaid's thermal reactor.

control of up to 54 sample tubes is achieved either by a thermocouple located in the block or in a control tube. Up to 25 programme protocols can be stored in the instrument's non-volatile memory and viewed on the LED display.

For the high-speed temperature changes required for DNA amplification, Violet of Italy recommends the Biostar **automatic thermal cycler** (*Reader Service No. 110*). Biostar uses solid-state technology with no valves or liquids, and can accommodate 25 0.5-ml or 16 1.5-ml cuvettes. The instrument has a temperature range of 0–100 °C, which is accurate to ±0.4 °C, says Violet. Typically, the instrument can change from 98 °C to 37 °C in 48 seconds, and from 32 °C to 72 °C in 35 seconds. Up to 40 protocols can be stored, which can include up to 99,999 cycles with steps timed to last between 0 and 9,999 seconds. The \$4,400 (US) Model BS-300 is supplied without a PC. Prices for Biostar if supplied with either an XT- or AT-compatible PC are \$5,850 and \$6,880 (US), respectively.

For reactions that require rapid temperature transitions, such as DNA

sequencing and enzyme analysis, LEP Scientific has introduced the PREM Series III **programmable heat sequencer** (*Reader Service No. 111*). The PREM Series III has an operating range of 15–105 °C and a refrigerator programme for the storage of samples between 4–8 °C at the end of the run. Additional design features include interchangeable incubation blocks for a range of tube sizes, microtitre plates and strips, the facility for remote control and the option of delayed start for overnight operation. Up to 20 programmes can be stored.

Detection systems

As an alternative to silver staining, Collaborative Research, Inc. is offering ElectroSep Gold Blot Stain for the **detection of proteins on Western blots** (*Reader Service No. 112*). The \$105 (US) gold blot stain provides protein visualization on PVDF and nitrocellulose membranes in 30–60 minutes with a sensitivity equal to or greater than silver staining of polyacrylamide gels, says the company. ElectroSep is supplied as a ready-to-use solution for staining 100 0.8 × 10-cm strips or 15 10 × 10-cm membranes.

Vector Laboratories has introduced the **PHOTOPROBE biotin labelling/detection kit** for biotinylating DNA/RNA probes and their detection on nitrocellulose/nylon membranes and tissue sections (*Reader Service No. 113*). Nucleic acid-

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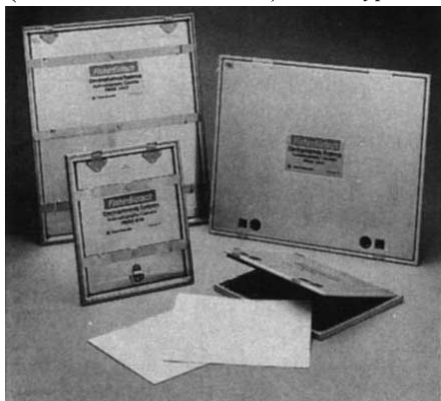
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are labelled with the light-sensitive Photoprobe biotin, which must be reconstituted before use and can be stored at -20°C for up to a year in this form, says Vector. Conditions for hybridization are, according to Vector, similar to those used with radioactively-labelled nucleic acids. Once hybridized to the nitrocellulose membrane, the probes can be detected with Vectastain ABC-AP reagent and visualized by the addition of alkaline phosphatase. A positive result is indicated by the presence of a brown/black precipitate. The £110 (UK) kit contains sufficient reagents to biotinyrate about 500 μg of DNA or RNA probe and stain about 25 100 cm^2 Northern or Southern blots.

A new line of **exposure cassettes and intensifying screens** for autoradiography are available from Fisher Scientific (Reader Service No. 114). Two types of



Autorad accessories from Fisher Scientific Instruments.

cassettes are available in either 8×10 -inch or 14×19 -inch sizes: the Series AC light-weight aluminium or Series XC stainless steel variety. The Series AC cassettes are designed with a fail-safe latch to prevent accidental opening and have felt-covered urethane foam pads to keep the film and screen in even contact. The XC Series can accommodate screens of any thickness because of the lead foil-lined door that is attached by self-adjusting floating hinges. Stainless steel locking bars provide a light-tight fit, even contact, and are easy to open, says Fisher.

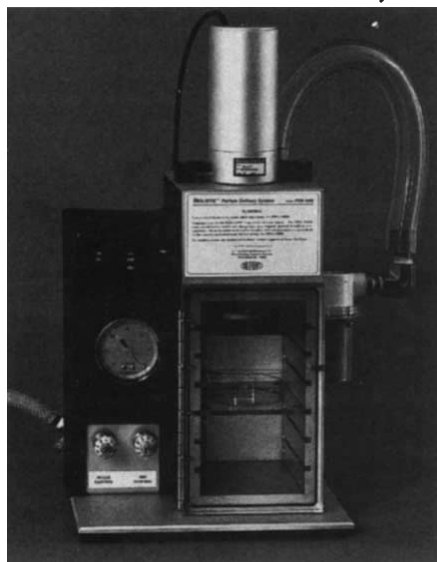
Tools for transfection

For the transfection of DNA and other molecules into mammalian, plant or bacterial cells, International Biotechnologies, Inc. has introduced the gene-ZAPPER 450/2500 **electroporation system** (Reader Service No. 115). The light-weight, microprocessor-controlled gene-ZAPPER comprises control and reaction modules that are made of acrylic plastic, allowing the user visually to detect when the pulse is delivered. The instrument has 34 capacitance settings and a voltage range of 50–2,500 V. Reusable metal electrodes or specially designed foil-lined cuvettes can be used, which provides



IBI's geneZAPPER electroporation system. flexibility and is cost effective, says IBI. Safety features of the instrument include recessed input and output jacks, two separate discharge buttons that require two-handed operation for triggering, a safety guard for the electrode assembly and proprietary circuitry that allows the unit automatically to discharge internally.

The Bolic PDS-1000 **particle delivery system** can inject genetic or biological substances directly into cells in less than one microsecond, says Du Pont Co. (Reader Service No. 116). Microcarriers coated with biological substances are accelerated at high velocity inside a vacuum chamber so that they penetrate thousands of cell walls and membranes simultaneously with



Transform cells and tissues with the Bolic PDS-1000 particle delivery system. an even distribution, says Du Pont. New design features of the machine include changes to the initiator mechanism, an interlock safety device, vacuum flow valves, push-button action controls, and a redesigned accelerator mechanism. Transforming cells and tissues in tact has, says Du Pont, been used successfully in agricultural and non-agricultural plants, yeasts, algae, organelles and mammalian cells.

Services and software

Isogene Bioscience is offering a new **custom RNA synthesis service** for any sequence up to 50 to 60 nucleotides in length, including mixed sites (Reader

Service No. 117). The synthetic RNA oligos are made on an automatic DNA/RNA synthesizer by the stepwise coupling of 'protected' monomers, which, according to Isogene, has a 97–99 per cent efficiency at each step. The RNA is shipped and stored with a protecting group bound to the 2'-OH of ribose, which renders it insensitive to enzymes. Using Isogene's deprotection kit, the protecting group can be removed, producing biologically active RNA. Typical applications for custom RNA oligos include RNA-protein interactions, ribozymes, DNA/RNA hybrids, rDNA technology and antisense studies.

Softshu has released Version 4.0 of the **Recombinant Toolkit — software for the analysis of nucleotide and amino acid sequences** (Reader Service No. 118). Designed for use with MS-DOS microcomputers, the \$250 (US) Recombinant Toolkit 4.0 is a menu-driven, cursor-controlled software package featuring a reverse translation with restriction facility for accurate gene design, cDNA analysis and the study of site-specific mutagenesis of proteins to create restriction sites. Restriction and open-reading frame analysis can be performed on up to 32-K nucleotide sequences, with data supplied for 211 restriction sites. Other features include a macro search language, full user control over selection of scales, window sizes and colour schemes for the presentation of amino acid sequences, on-line help, built-in NOTEPAD, and Genbank, Stanford, Deyhoff or ASCII format compatibility. □

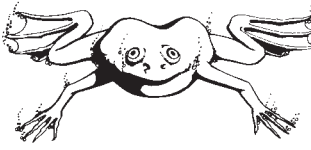
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